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Efeitos tóxicos do ácido acetilsalicílico e seus principais metabolitos em espécies padrão e autóctones

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resumo

A presença de resíduos farmacológicos em sistemas aquáticos tornou-os um factor de contaminação preocupante, principalmente devido à sua capacidade para interferir em sistemas biológicos específicos.

Assim, neste trabalho pretendeu-se avaliar os efeitos induzidos pela substância activa da Aspirina® - ácido acetilsalicílico (ASA) – e seus principais produtos de metabolização – ácido salicílico (SAL), ácido gentísico (GEN) e ácido *o*-hidroxihipúrico (HDP), também denominado como ácido salicilúrico - em organismos aquáticos não alvo. Neste sentido, desenvolveram-se ensaios agudos e crónicos em que as respostas da espécie padrão (*Daphnia magna*) e autóctone (*Daphnia longispina*) de cladóceros foram comparadas. Para além disso, testou-se a inibição aguda da bioluminescência da bactéria marinha *Vibrio fischeri*.

Os resultados obtidos evidenciaram que a maior parte dos compostos testados induziram efeitos nocivos nos organismos de teste utilizados. Somente ASA e SAL, nas soluções de teste de concentração mais elevada, inibiram a bioluminescência de *V. fischeri*. Contudo, verificou-se a imobilização de cladóceros de acordo com o grau de toxicidade GEN > ASA > SAL > HDP. Este último não revelou toxicidade aguda em ambos os cladóceros, para concentrações inferiores ao seu limite de solubilidade. A espécie autóctone mostrou-se mais sensível do que a padrão perante exposições agudas aos tóxicos.

Nos ensaios crónicos, de uma forma geral, observou-se a mesma sensibilidade relativa entre as duas espécies. No entanto, a taxa de crescimento diária de *D. magna* foi mais afectada do que a de *D. longispina*, na presença de ASA, SAL e GEN. Para além disso, o cladócero autóctone manifestou uma plasticidade fenotípica mais desenvolvida, estimulando a taxa de crescimento intrínseco da sua população (*r*), quando sujeito a ASA ou a SAL. Não obstante, tanto GEN como HDP inibiram o *r* nas duas espécies de cladóceros, devido à mortalidade e redução da fecundidade normal das fêmeas a eles expostas. HDP, em particular, promoveu a produção de descendência inviável, principalmente representada pelo elevado número de ovos abortados por *D. magna* e *D. longispina*. Nos ensaios crónicos GEN tendeu a ser o composto mais tóxico, seguido de ASA, SAL e HDP para ambos os cladóceros.

Em suma, não só o ingrediente activo da Aspirina® interfere na sobrevivência e ciclo de vida de organismos não alvo, mas também os seus metabolitos, tendo sido GEN o composto mais tóxico. Os testes agudos mostraram-se insuficientes para avaliar a toxicidade de HDP, a qual se manifestou ao nível da reprodução dos cladóceros após exposições crónicas. A utilização de espécies autóctones permite inferir acerca do comportamento individual e populacional de organismos sujeitos a condições desfavoráveis no seu ambiente natural.

abstract

The presence of pharmacological residues in aquatic systems led them to be an important contamination issue, mainly due to their ability of interfering in specific biological systems.

Therefore, the present work aimed to study and compare the toxic effects of Aspirin® active ingredient – acetylsalicylic acid (ASA) - and its main metabolites – salicylic acid (SAL), gentisic acid (GEN) and *o*-hydroxyhippuric acid (HDP), also named as salicyluric acid – on survival, reproduction and growth of *Daphnia magna* (a standard species) and *Daphnia longispina* (an autochthonous species). Moreover, it was tested the acute bioluminescent inhibition of the marine bacterium *Vibrio fischeri*.

Our results showed that the majority of the tested compounds induced negative effects on test organisms. Neverthless, only ASA and SAL, at the highest concentrations of test solutions, inhibited *V. fischeri* bioluminescence. The sequence of decreasing toxicity for both cladocerans was GEN > ASA > SAL > HDP, being the latter the least toxic as it did not induce daphnids immobilization to concentrations below its solubility limit. The autochthonous species was the most sensitive one.

In general, in chronic assays, the same sensibility trend was observed for both species. In spite of this, the daily growth rate of *D. magna* was more affected than that of *D. longispina* under ASA, SAL and GEN exposures. Furthermore, the autochthonous cladoceran stimulated its population growth (*r*) when subjected to ASA or SAL, which probably reflects an increased phenotypic plasticity. Notwithstanding, GEN and HDP inhibited both daphnids *r*, due to females mortality and reduction of their normal fecundity. In particular, HDP enabled the production of inviable offspring, mainly represented by the increasing egg abortion numbers by *D. magna* and *D. longispina*. Overall, GEN was, generally, the most toxic compound, followed by ASA, SAL and HDP.

Thus, the active ingredient of Aspirin® and its metabolites interferred in non-target organisms survival and life-history traits, being GEN the most toxic compound. The acute assays showed to be insufficient to assess HDP toxicity, which impared the normal reproduction of cladocerans during long-term exposures. Autochthonous species seem to give a more feasible and ecological relevant understanding about their behaviour and population dynamics under chronic environmental agressions.

Um especialista é aquele que
cometeu todos os erros possíveis
numa matéria muito concreta.

Niels Henrik Davis Bohr
(1885-1926)

À Mimi

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Introdução geral

INTRODUÇÃO GERAL

1. Resíduos farmacológicos - contaminantes do meio aquático

O desenvolvimento exponencial das sociedades actuais, aliado ao consumo exacerbado, tem contribuído para o declínio da qualidade ambiental. A introdução de compostos químicos, muitas vezes com impacto ecológico desconhecido, pode levar, a curto ou a longo prazo, a alterações nos ecossistemas naturais. O meio aquático, em particular, constitui o destino de diversos compostos químicos, quer por descargas directas, quer por deposição atmosférica ou lixiviação e escorrências a partir de terrenos contaminados. Deste modo, torna-se alvo de diversos estudos ecotoxicológicos que, em última instância, poderão contribuir para a compreensão de potenciais efeitos nefastos na saúde humana.

Até há poucos anos atrás este tipo de estudos limitava-se à análise de poluentes considerados prioritários (*e.g.* pesticidas, herbicidas, organoclorados persistentes), contudo, recentemente surgiu uma preocupação crescente relativa ao impacto de resíduos farmacológicos no meio aquático (Stan e Heberer, 1997; Ternes, 1998; Halling-Sørensen *et al.*, 1998; Daughton e Ternes, 1999; Stumpf *et al.*, 1999).

Os fármacos, à semelhança de outros xenobióticos (*i.e.*, compostos estranhos aos organismos), apresentam propriedades físico-químicas específicas que determinam a sua degradação, persistência, mobilidade, biodisponibilidade e bioacumulação nos organismos (Halling-Sørensen *et al.*, 1998; Jørgensen *et al.*, 2000). Na verdade, o seu elevado consumo (na Europa, alguns medicamentos ultrapassam as 100 toneladas/ano) (CSTEE, 2001) contribui para a contínua introdução destes compostos no ambiente, tornando-os, assim, "pseudo-persistentes" e ubíquos em diferentes ecossistemas (Daughton e Ternes, 1999; Zuccato *et al.*, 2000).

Embora a análise de diferentes amostras ambientais tenha evidenciado a presença de baixas concentrações de fármacos (desde ng/L a mg/L) (Jones *et al.*, 2001), o facto de serem compostos que interferem em sistemas biológicos específicos (*e.g.*, receptores membranares, actividades enzimáticas, cascatas de sinalização) podem, pelo seu largo espectro de acção, induzir alterações em organismos não alvo (Zwiener e Frimmel, 2000).

Desta forma, torna-se relevante desenvolver estudos ecotoxicológicos que interpretem não só efeitos ao nível do indivíduo, mas também ao nível da estabilidade populacional, permitindo avaliar consequências de contaminações agudas e, principalmente, de exposições crónicas (Seiler, 2002).

Neste contexto, têm sido elaboradas algumas normas legislativas, embora a presença e o efeito de fármacos no ambiente seja ainda um tema em prospecção.

Em 1965, a Comunidade Económica Europeia começou a regulamentar o registo de propriedade de produtos médicos através da Normativa 65/65/EEC (CEEC, 1965 *in* Straub, 2002). Com a crescente preocupação da protecção do ambiente, em 1993 é aprovada a Normativa 93/39/EEC, em que é sublinhada a necessidade de se avaliar potenciais riscos ambientais face à aplicação/utilização de fármacos (CEEC, 1993a *in* Straub, 2002). No mesmo ano, são publicados procedimentos detalhados para o registo de novos medicamentos, incluindo a obrigatoriedade da sua Análise de Risco Ambiental (ARA) (Dec. Lei nº 2309/93/EEC; CEEC, 1993b *in* Straub, 2002).

Entretanto, em publicações mais recentes, orientadas pela EMEA (Agência Europeia para Avaliação de Medicamentos), foram aperfeiçoadas directrizes para desenvolver a ARA de fármacos; distinguiram-se grupos de substâncias com e sem organismos geneticamente modificados, dentre os quais se dissociou ARA para medicamentos de uso veterinário (EMEA, 1998 *in* Koschorreck *et al.*, 2002) e para medicamentos de uso humano (CSTEE, 2001).

1.1. Vias de exposição, destino, efeitos e ocorrência no ambiente

Desde finais da década de 70 tem-se vindo a assistir ao desenvolvimento de esforços no sentido de determinar o transporte, destino, ocorrência e efeitos de fármacos no ambiente (Halling-Sørensen *et al.*, 1998; Daughton e Ternes, 1999; Jørgensen *et al.*, 2000; Jones *et al.*, 2001; Kümmerer, 2001a). No entanto, as noções adquiridas revelam-se ainda pouco abrangentes nesta matéria. Este facto pode ser não só atribuído à limitação dos métodos analíticos para detectar concentrações muito baixas em matrizes ambientais complexas (Kolpin *et al.*, 2002), mas também à multiplicidade de substâncias activas, que,

uma vez no ambiente, exibem comportamentos farmacodinâmicos e farmacológicos díspares (Länge e Dietrich, 2002).

Através da excreção metabólica (3 e 13), da rejeição de medicamentos não consumidos (4) e da emissão de resíduos provenientes dos locais de produção (1) de fármacos torna-se, então, possível a entrada de diversos compostos químicos nos ecossistemas, contribuindo para a contaminação do meio aquático (Figura 1) (Zuccato *et al.*, 2000).

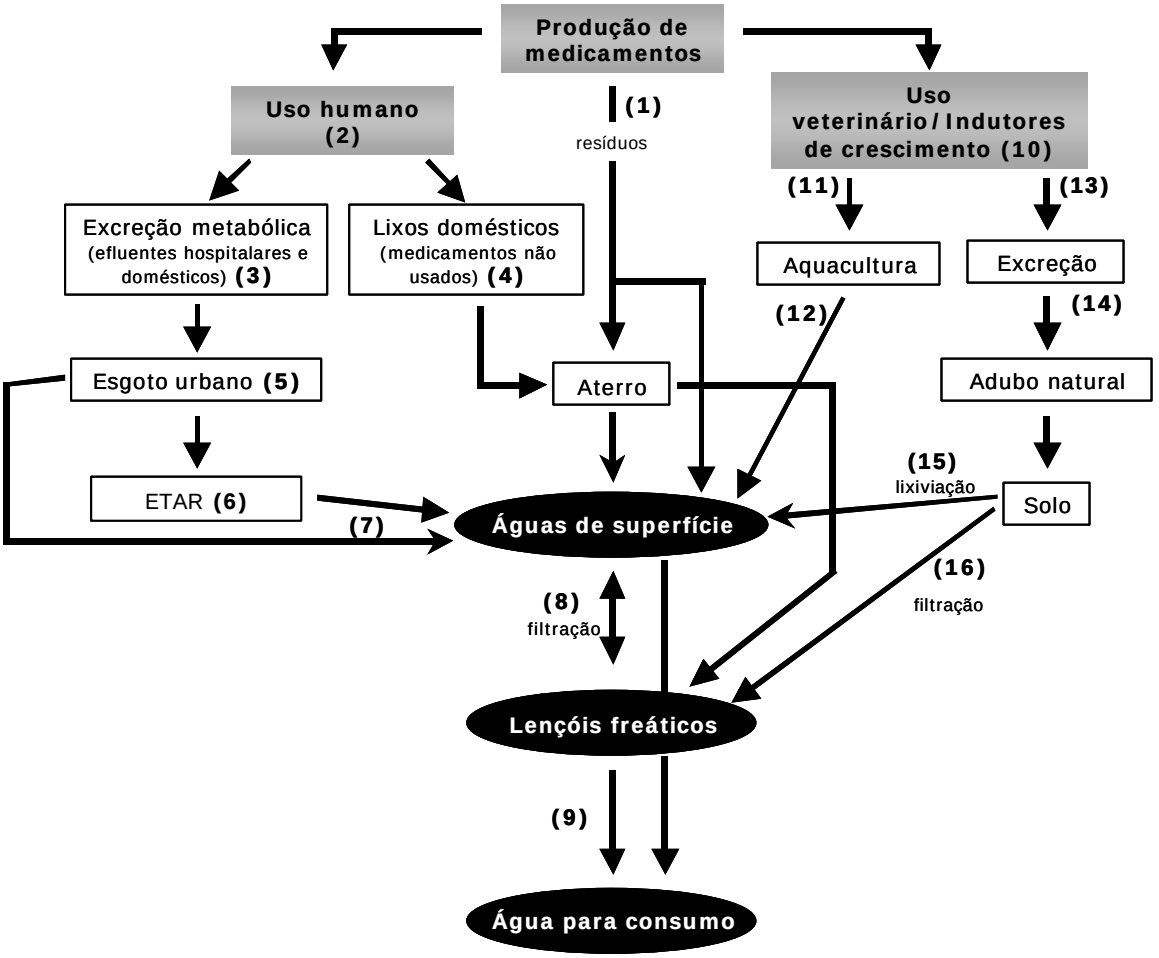

Fig. 1. Possíveis vias de exposição e distribuição de resíduos farmacológicos no ambiente. [Adaptado de Halling-Sørensen *et al.*, 1998; Daughton, 2001; Alder *et al.*, 2001; Heberer, 2002a].

Grande percentagem dos compostos usados no tratamento de doenças humanas (2) são apenas ligeiramente transformados ou mesmo inalterados,

sendo a menor percentagem excretada sob a forma de metabolitos (Heberer, 2002a). Em qualquer dos casos, são eliminados pela urina e/ou fezes, terminando em efluentes domésticos e hospitalares (3), os quais serão conduzidos para um esgoto urbano conjunto (5), posteriormente tratado em ETARs (estações de tratamento de águas residuais) (6). No decorrer destes processos de limpeza e desinfecção, os fármacos e metabolitos podem ser completamente biodegradados; se possuírem propriedades lipofílicas acentuadas ficam adsorvidos a partículas; se forem persistentes e hidrofílicos, não sofrem degradação nem remoção durante o tratamento de água, atingindo os sistemas aquáticos naturais (7) (Halling-Sørensen *et al.*, 1998; Sedlak *et al.*, 2001). A posterior lixiviação de compostos depositados nos sedimentos de águas superficiais promove a contaminação difusa de lençóis freáticos subterrâneos (8), dos quais, muitas vezes, se canaliza água para consumo humano (9) (Heberer, 2002a).

Os medicamentos veterinários (10) são administrados não só com um fim preventivo e/ou terapêutico, mas também para estimular o crescimento dos organismos em agropecuária e em sistemas de aquacultura (11) (Stan e Heberer, 1997; Haller *et al.*, 2002). Assim, a descarga directa de efluentes de estações de aquacultura, contendo grande quantidade de compostos não ingeridos pelos peixes e metabolitos de excreção, num ecossistema aquático (12), poderá afectar os organismos nele presentes (Halling-Sørensen *et al.*, 1998). Por outro lado, a rejeição de substâncias metabólicas através dos dejectos animais (13), lançados directamente nos locais de pastagem ou reaproveitados como adubo natural para campos de cultivo (14), podem atingir águas superficiais por escorrências dos terrenos (15). Além disso pode ainda ocorrer a lixiviação de substâncias através do solo, contribuindo para a contaminação dos lençóis freáticos (16) (Kümmerer, 2001b).

Em suma, os fármacos assumem uma presença constante e uma larga distribuição no ambiente aquático. Aliás, vários estudos efectuados na Europa e no continente americano provam a ubiquidade destes poluentes em efluentes urbanos (Hartig *et al.*, 1999), em efluentes de ETARs (Desbrow *et al.*, 1998; Routledge *et al.*, 1998; Ternes, 1998; Ternes *et al.*, 1999), em águas de superfície (Ternes *et al.*, 1998; Zuccato *et al*., 2000; Alder *et al.*, 2001; Lindsey *et al.*, 2001; Ternes *et al.*, 2001; Kolpin *et al.*, 2002), em lençóis freáticos (Heberer *et al.*, 1997; Sacher *et al.*, 2001; Heberer *et al.*, 2001) e em água para

consumo humano (Heberer, 2002a; Heberer *et al.*, 2002; Reddersen *et al.*, 2002).

Ainda que em baixas concentrações, os fármacos podem interferir com o equilíbrio dos organismos, induzindo possíveis efeitos subtis, cuja acumulação ao longo do tempo venha a manifestar profundas alterações passíveis de serem confundidas e aliadas à variabilidade natural dos organismos (Daughton, 2000).

Na verdade, estudos recentes denunciaram a acção negativa de disruptores endócrinos em diferentes invertebrados aquáticos (Barry e Stoopman, 2000; Andersen *et al*., 2001; Hugget *et al.*, 2002; Hutchinson, 2002) e em peixes (Routledge *et al.*, 1998; Länge *et al.*, 2001; Zerulla *et al.*, 2002; Schmid *et al.*, 2002). Por outro lado, foi também avaliado o efeito de antibióticos, entre outros medicamentos, em plantas aquáticas (Migliore *et al.*, 2000), em dafnídeos (Lilius *et al.*, 1995; Wollenberger *et al.*, 2000) e nalguns invertebrados terrestres (Baguer *et al.*, 2000); assim como o efeito genotóxico de efluentes hospitalares foi comprovado por meio de testes agudos bacterianos simples (Hartmann *et al.*, 1998).

1.2. Aspirina® e seus principais metabolitos

Em 1880, Felix Hoffman sintetizou pela primeira vez, a partir do ácido salicílico (SAL; ácido di-hidroxibenzóico), o ácido acetilsalicílico (ASA; ácido 2-(acetiloxi)benzóico), o qual veio a constituir o princípio activo da Aspirina®, patenteada em 1897 pela Bayer and Company (Bayer[1]).

Actualmente, o ASA continua a encontrar larga aplicação em diferentes quadros terapêuticos. Normalmente é utilizado como analgésico, antipirético ou anti-inflamatório (Kamanyire, 2002), apesar de ser também indicado para o tratamento de doenças reumatológicas (Akaike *et al*., 2002) e cardiovasculares (ASHP, 1997) (devido às suas propriedades anti-coagulantes), estando ainda a ser estudada a sua potencial aplicação como agente quimioterapêutico, nomeadamente no tratamento de cancro de cólon (Reuter *et al.*, 2002).

Dado o seu largo espectro de acção, a Aspirina® é anualmente consumida em grandes quantidades. De acordo com dados fornecidos pelo INFARMED (Instituto Nacional da Farmácia e do Medicamento), em 2001, o SNS (Serviço Nacional de Saúde) vendeu, em ambulatório, 969.915 embalagens de Aspirina®,

[1] Informação retirada do site http://www.aspirin.com/world of aspirin en.html#.

não incluindo a quantidade administrada nos serviços hospitalares ou vendida sem prescrição médica.

Após administração oral, o ASA absorvido ao longo do tubo digestivo (80-100%) (Schulman *et al.*, 2002) é praticamente hidrolisado a SAL (10-85%), o principal metabolito no plasma sanguíneo, responsável pela maior parte dos efeitos terapêuticos deste medicamento (Henschel *et al.*, 1997). O SAL sofre posterior metabolização: parte é conjugado com glicina, originando o ácido *o*-hidroxihipúrico (HDP; também conhecido como ácido salicilúrico; glicina-2-hidroxibenzoílo), e parte é hidroxilado a ácido gentísico (GEN; ácido 2,5-dihidroxibenzol). Não obstante, estes dois metabolitos formam-se em menor quantidade (HDP: cerca de 10%; GEN: cerca de 4%) (DeBlassio *et al.*, 2000; Schmid *et al.*, 2001; Zaugg *et al.*, 2001).

Mais de 90% da substância inicialmente absorvida é eliminada na urina, quer sob a forma livre, quer sob forma de metabolitos, ocorrendo a possibilidade destes se reconverterem no composto original, enquanto que o ASA excretado pode ser biodegradado nos seus metabolitos (Henschel *et al.*, 1997). De acordo com estudos desenvolvidos, HDP foi o metabolito detectado em maior percentagem na urina de humanos (60-75%) (Patel *et al*., 1990; DeBlassio *et al.*, 2000).

Apesar de Richardson and Bowron (1985) terem evidenciado que o ASA e seus metabolitos eram praticamente biodegradados durante os processos de tratamento de água, estudos mais recentes referem a sua presença em efluentes de ETARs e em amostras de águas de superfície: ASA, concentrações máximas de 3,0-3,1 ?g/L (Richardson and Bowron, 1985; Halling-Sørensen *et al.*, 1998; Ternes *et al.*, 1998; Stumpf *et al.*, 1999; Schulman *et al.*, 2002); SAL, concentrações máximas de 4,1-95,62 ?g/L (Hignite and Azarnoff, 1977; Ternes, 1998; Ternes *et al.*, 1998; Heberer *et al.*, 2001; Heberer, 2002b); GEN, concentrações máximas de 0,59-1,2 ?g/L (Ternes, 1998). HDP não foi determinado em amostras de água tratadas (Ternes, 1998).

Como já foi referido, os efeitos tóxicos que advêm da exposição dos organismos a compostos farmacológicos foram ainda pouco estudados e, além disso, os dados que existem referem-se principalmente à sua toxicidade aguda, limitando-se ao estudo das substâncias activas dos medicamentos. Contudo, a biotransformação destas substâncias pode contribuir para a formação de metabolitos que apresentem um grau de toxicidade muito mais elevado do que o

composto original. Desta forma, torna-se relevante realizar testes ecotoxicológicos que visem avaliar os efeitos agudos e crónicos de substâncias activas e seus metabolitos.

2. Vibrio fischeri e *Daphnia* sp. - organismos de teste em ecotoxicologia

Em estudos de ecotoxicologia é prática comum recorrer à aplicação de testes ecotoxicológicos agudos e crónicos para analisar o efeito de compostos químicos no ambiente (ASTM, 1980).

Neste âmbito, utilizam-se organismos de diferentes níveis tróficos, sensíveis à potencial acção toxicológica do xenobiótico, podendo, assim, manifestar um determinado efeito durante o período de exposição estabelecido.

A bactéria marinha bioluminescente *Vibrio fischeri* (Beijerinck) Lehmann et Neumann é um dos organismos usados para avaliar a toxicidade aguda de compostos químicos em ecotoxicologia. A redução da intensidade da luminosidade reflectida é medida através do teste vulgarmente denominado de Microtox®. Contrariamente a outros ensaios, este é um teste de rápida execução, que não exige manutenção de organismos em laboratório (a suspensão de bactéria é congelada e conservada durante um período relativamente longo) (Kaiser, 1998), apesar do seu custo de aquisição e de manutenção ser muito elevado.

Daphnia sp. (Cladocera; Branchiopoda; Crustacea), embora necessite de contínua manutenção em laboratório, exige métodos simples com um valor económico reduzido. Para além disso, este organismo filtrador possui um ciclo de vida curto, ao longo do qual, sob condições favoráveis, se reproduz assexuadamente por partenogénese, produzindo uma descendência relativamente grande, cuja variabilidade genética é reduzida (Barata *et al.*, 2000). Daí que diferentes espécies de *Daphnia*, com diferentes sensibilidades a compostos químicos sejam estudadas e utilizadas como organismos de teste, quer em ensaios agudos, em que o parâmetro a analisar é a imobilização de indivíduos, quer em ensaios crónicos, nos quais a inibição da reprodução normal e a variação da taxa de crescimento são os parâmetros de avaliação de uma possível acção toxicológica. Aliás, Lilius *et al.* (1995) referem que os testes de toxicidade com invertebrados têm sido propostos como uma potencial alternativa para extrapolar efeitos tóxicos em animais superiores.

Daphnia magna Straus e *Daphnia longispina* O.F. Müller são duas espécies de cladóceros frequentemente aplicadas em estudos deste tipo (Lilius *et al.*, 1995). *D. magna* é uma espécie padrão, vulgarmente utilizada em ecotoxicologia já que garante coerência e repetibilidade de resultados entre diferentes laboratórios (Soares, 1989). *D. longispina*, por seu lado, é uma espécie autóctone de menores dimensões, fazendo parte da comunidade zooplanctónica de lagoas, nomeadamente, da zona Litoral Centro de Portugal (Barros, 1994; Pereira, 1997; Abrantes, 2002), tendo já sido utilizada em testes laboratoriais e de campo para avaliar o estado de um sistema (Ojala *et al.*, 1995; Antunes *et al.*, em publicação).

O efeito de fármacos em *Daphnia* spp., nomeadamente em *D. magna*, já foi alvo de alguns estudos ecotoxicológicos, tendo sido realizados ensaios agudos (Lilius *et al.*, 1995; Cleuvers, 2002; Huggett *et al*., 2002) muitas vezes incluídos em estudos de ARA (Henschel *et al.*, 1997; van Wezel e Jager, 2002). Também têm surgido, embora em número muito reduzido, estudos que avaliam a exposição crónica destes organismos a compostos farmacológicos (Barry e Stoopman, 2000; Wollenberger *et al.*, 2000; Hutchinson, 2002; Ribeiro *et al.*, 2002).

3. Objectivos

Os objectivos delineados para o desenvolvimento do presente trabalho consistiram em:

- estudar os efeitos induzidos pela substância activa da Aspirina® (ácido acetilsalicílico) e seus principais metabolitos (ácido salicílico, ácido gentísico e ácido hidroxihipúrico) em dois cladóceros – *Daphnia magna* e *Daphnia longispina* - (efeitos agudos e crónicos), e numa bactéria – *Vibrio fischeri* - (efeitos agudos);

- comparar as respostas de uma espécie de cladócero padrão (*D. magna*) com a de uma espécie autóctone (*D. longispina*), expostas àqueles compostos químicos;

- inferir acerca da sensibilidade dos parâmetros biológicos analisados para avaliar o impacto de substâncias farmacológicas em *Daphnia* sp.

4. Estrutura da dissertação

Esta dissertação foi dividida em quatro partes. No capítulo I descrevem-se vias de entrada, destino e ocorrência de resíduos farmacológicos no ambiente. Refere-se em particular a Aspirina® e seus principais metabolitos, como potenciais xenobióticos com acção toxicológica sobre organismos não alvo, sujeitos a exposições agudas ou crónicas. Neste capítulo definem-se, ainda, os objectivos desta dissertação.

Nos capítulos II e III apresentam-se os resultados obtidos em ensaios agudos e crónicos em que se testou, respectivamente, o ácido acetilsalicílico (princípio activo da Aspirina®) e os seus principais metabolitos, numa bactéria – *V. fischeri* – e em duas espécies de cladóceros – *D. magna* e *D. longispina*. Estes dois capítulos constituem artigos submetidos a revistas da especialidade.

Por fim, no capítulo IV apresenta-se a discussão geral dos resultados obtidos, inferindo acerca do potencial impacto deste tipo de resíduos em organismos chave em ecossistemas aquáticos.

Capítulo II

Life-History Traits of Standard and Autochthonous Cladocerans:
I - Acute and Chronic Effects of Acetylsalicylic Acid

Life-History Traits of Standard and Autochthonous Cladocerans:
I - Acute and Chronic Effects of Acetylsalicylic Acid

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Submited to Chemosphere

ABSTRACT

Pharmaceuticals have been recognised as an important group of aquatic micropollutants, mainly due to their biological active nature. Acetylsalicylic acid (ASA), which is the active compound of Aspirin® and many other pharmaceuticals, is largely consumed every year. Therefore, acute and chronic effects were investigated on standard (*Daphnia magna*) and autochthonous (*Daphnia longispina*) daphnids, and it was also assessed the bioluminescence inhibition of *Vibrio fischeri*. Results showed that ASA impared the survivorship, reproduction and growth of cladocerans species, and inhibited the bioluminescence of *V. fischeri* (EC_{50} for the concentrations of 1000 and 1800 mg/L were, respectively, 878.64 and 445.48 mg/L). The standard daphnid was the most tolerant species in acute assays (EC_{50}-48h = 1293.05 mg/L; *D. longispina*: EC_{50}-48h = 647.31 mg/L); while the autochthonous one seemed to be more resistant under ASA chronic exposures mainly at population-level traits. In spite of this, the obtained effect concentrations were much more higher than ASA environmental concentrations. Notwithstanding, the impairment of individual-level traits are likely to occur under this environmental levels as an ultimate response, after long term exposures.

Key words: pharmaceuticals, acetylsalicylic acid, *Daphnia magna*, *Daphnia longispina*, *Vibrio fischeri*, bioluminescence.

INTRODUCTION

Several authors have already reported the occurrence of pharmaceuticals in the aquatic environment (*e.g.*, Hignite and Azarnoff, 1977; Stan and Heberer, 1997; Halling-Sørensen *et al.*, 1998; Hirsch *et al.*, 1999; Stumpf *et al.*, 1999; Ahrer *et al.*, 2001; Sacher *et al.*, 2001; Haller *et al.*, 2002; Kolpin *et al.*, 2002). Due to their biological active nature, pharmaceuticals have been recognised as an important group of aquatic micropollutants, although, only in the past few years they have become a major issue in the environmental chemistry and toxicology (Zwiener and Frimmel, 2000; Heberer, 2002).

Every year large amounts of pharmaceuticals are used in human and veterinary medicine (Stan and Heberer, 1997; Ternes, 1998; Daughton and Ternes, 1999; Zwiener and Frimmel, 2000), in agriculture and aquaculture as grown additives (Hirsch *et al.*, 1999). Therefore, drugs residues may enter the water cycle coming from point discharges of manufacturers, direct disposal of unused medication into domestic sewage, excretion of unchanged or metabolized pharmaceuticals after human and/or animal intake, runoff of soils in which wastewater or sewage sludge's manure was dispersed and, finally, effluents from aquaculture (Hirsch *et al.*, 1999; Stan and Heberer, 1997; Daughton and Ternes, 1999; Zwiener *et al.*, 2000).

Acetylsalicylic acid (ASA; 2-(acetyloxy)benzoic acid) (Fig. 1), which is the active ingredient of Aspirin® and of many other pharmaceuticals, is one of the most widely prescribed analgesics in human medical care, often sold in high quantities as an over-the-counter (OTC) drug (Heberer, 2002). In Portugal, a rough estimative provided by the INFARMED (National Institute of Pharmacy and the Pharmaceutical) indicated that NHS (National Health Service) sold 969915 Aspirin® packages of 500 mg in 2001 (it does not include those amounts given as medication in hospitals or sold as OTC drugs).

Usually, after oral administration, ASA will overwhelm hepatic metabolic reactions which transform it into conjugates (*e.g.*, glucoronides) more reliably excreted. However, a significant amount of parent compound will be released without any changes, and it will thereafter enter raw sewage or manure. Subsequently, the probability of surface, ground and drinking water contamination will be considerably high. Indeed, many authors (*e.g.*, Richardson and Bowron, 1985; Stumpf *et al.*, 1996 *in* Halling-Sørensen *et al.*, 1998; Ternes

et al., 1998; Schulman *et al.*, 2002) have already reported the presence of ASA on sewage effluents and surface waters at maximum loading levels of >3.1 ?g/L and 1.5 ?g/L, respectively.

Hence pharmaceuticals often occur in low concentrations (Ternes, 1998) and, generally, they are highly specific and potent in their effects, specially in non-target organisms (Henschel *et al.*, 1997). Therefore, it should be evaluated whether pharmaceuticals induce subtile hardly undetected and irreversible effects on animals subjected to long term exposures or not. Thus, the present work aims to study and compare the toxic effects of ASA on survival, reproduction and growth of *Daphnia magna* Straus (a standard species) and *Daphnia longispina* O.F. Müller [an autochthonous species with high densities and large distribution in Portuguese shallow lakes (Barros, 1994; Pereira, 1997; Abrantes, 2002)]. Moreover, it is also determined the acute toxicity of ASA on *Vibrio fischeri* (Beijerinck) Lehmann et Neumann, a bioluminescent bacterium used to conduct Microtox? test.

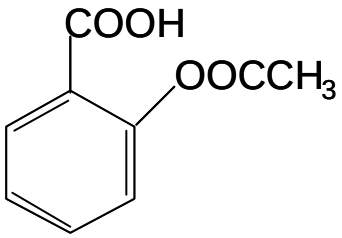

Fig. 1. Chemical structure of ASA.

MATERIAL AND METHODS

Daphnid Culture

Parent animals were reared in 800 ml of ASTM hard water (ASTM, 1980; EPA, 1989) (hereinafter referred as ASTM) with an organic additive made of *Ascophylum nodosum* (L.) Le Joli seaweed extract (Baird *et al.*, 1988), which was prepared by dilution of a stock solution (Soares, 1989), and added to the culture medium at a concentration of 6 ml/L. Individuals were fed every two days with

Pseudokirchneriella subcapitata Korshikov cultured in the lab (Stein, 1973) (3.00 x 10^5 and 1.50 x 10^5 cell/ml/*Daphnia* for *D. magna* and *D. longispina*, respectively). The cultures were maintained with no aeration supply, 16^L:8^D photoperiod and a temperature of 20 ? 1ºC.

Chemicals and Test Solutions

Test solutions of ASA (99.5% pure; obtained from Sigma-Aldrich Chemie?, Germany) were obtained by dilution of a stock solution, which was prepared with ASTM hard water previously to the test performance. The pH of the stock solution was adjusted to the 6-9 range. During the experimental chronic period the ASA stock solution was stored at 2-8ºC in dark. After a preliminary test, the chosen concentrations of ASA for the acute immobilization test were within the range 900.00-2350.00 mg/L for *D. magna* and 347.00-900.00 mg/L for *D. longispina*; while the reproduction assay was performed at 1.00, 1.80, 3.20, 5.60 and 10.00 mg/L for both cladocerans species. *V. fischeri* was subjected to ASA concentrations between 180.00-1800.00 mg/L.

Experimental Design

1. Tests with Daphnids

Tests were conducted according to OECD guidelines for *Daphnia* sp. Acute Immobilization Test (OECD, 2000), and *D. magna* Reproduction Test (OECD, 1996), being the last one adapted for *D. longispina*. All experiments were carried out with a single clone of *D. magna* (clone A *sensu* Baird *et al.*, 1989) and *D. longispina* (EM7 clone *sensu* Antunes *et al.*, in press). In order to minimize maternal effects, only neonates from the third to the fifth broods ageing less than 24h were tested (Barata and Baird, 1998).

Immobilization Test. Daphnids were mantained in 180 ml glass vessels for 48h, with 50ml of appropriate volumes of ASTM and test solutions. Five animals were randomly assigned in each vessel, with 4 replicates per treatment. Incubation conditions were the same as those described for the culture regime with the exception that animals were not fed. The dissolved oxygen (Oxi 330 WTW) and pH (pH 330 WTW) were measured in the beginning and at the end of the test.

After 48h the number of immobilized organisms was recorded, after shaking the vessel smoothly.

Reproduction Test. Ten replicates were filled with appropriate volumes of ASTM and test solutions. Daphnids used in 21-day-chronic assay were individually cultured in 50 ml glass vessels performed for the control and for each tested ASA concentration. Experimental conditions were similar to those already described above for the daphnids culture, including the feeding regime. Test organisms were transferred to newly prepared test solutions three times a week. Oxygen concentration and pH levels in vessels were measured weekly to guarantee that they were not limitting factors of biological responses. During the test, the parameters recorded to evaluate reproduction were: total number of neonates, number and size of broods, average size of three randomely chosen neonates from the first brood and age at each brood release. Growth was assessed by measuring the length (Olympus SZX9 esteromycroscope with an ocular micrometer) of the first exopodite of the second antennae (AL) of the first moult, and the last moult released within 21 days. The mortality of the parent animals was also daily observed. *D. magna* and *D. longispina* body length (BL) was obtained from two allometric relations: regression (1) was obtained by measuring the AL and BL of 416 *D. magna* individuals during their life cycle (unpublished data), while the second one (2) was determined by Silva (1999).

$$BL_{D.magna} = 10.558 \times AL_{D.magna} - 0.3475 \ (mm) \ (r^2 = 0.9615) \quad (1)$$

$$BL_{D.longispina} = 10.5 \times AL_{D.longispina} - 0.1437 \ (mm) \ (r^2 = 0.9621) \quad (2)$$

Daily growth rate (DGR) was determined by (3):

$$DGR = [\ln(BL_f) - \ln(BL_i)] / ?t \quad (days^{-1}), \quad (3)$$

where, BL_f is the final organism body length (mm) after 21 days, BL_i is the initial body length (mm) and ?t is the time interval (days) (Sobral, 1997; Burns, 2000). On the other hand, it was also calculated the rate of increase of the population (*r*, day^{-1}) by means of the Euler-Lotka equation (4):

$$\sum e^{-r.x}.l_x.m_x = 1 \qquad (4)$$

where, x is the age class (days; 0...n), l_x is the probability of surviving at age x and m_x the fecundity at age x. Standard deviation was determined according to Jackknife technique (Meyer *et al.*, 1986).

2. Microtox® Test

The Microtox® test (Microbics Corporation Inc. Protocols, 1988) is based on the inhibition of the luminescence of the marine bacterium *Vibrio fischeri* after 30 minutes exposure to the toxicant (Kaiser, 1998; Froehner *et al.*, 2000). In this case, a Basic Test Protocol (Microbics M500 Toxicity Analyzer) was carried out, in which *V. fischeri* was subjected to several dilutions of each ASA nominal concentrations.

Data Analysis

From each acute immobilization test, the number of immobilized organisms was plotted against the tested concentrations, and a 48-h EC_{50} at a 95% confidence limit (CL) was calculated by the standard probit procedure (Finney, 1971).

Mortalities that occurred during the chronic exposure were analysed by Fischer's exact test (EPA, 1989). No observed effect concentrations (NOEC) and lowest-observed-effect concentrations (LOEC) for reproduction and growth were achieved using one-way ANOVA, followed by a Dunnett´s test for multiple comparisons of the individual concentration effect and that of the controls (Zar, 1996). Non-normal or heterocedastic data were transformed using the square or log transformation. A Kruskal-Wallis test was run, followed by the Dunn's test (testing treatments *versus* control), whenever it was impossible to have normal and homocedastic data. Overall, a statiscally significant difference in reproduction or growth is reported for $P<0.05$. Females which died before the end of the test, including their offsprings, were excluded from the statistical analysis.

Microtox® EC_{50} results together with their 95% CLs were determined by the Microtox Software – Microbics Corporation MTX.EXE Version 6.01.

RESULTS

1. Acute Tests

In the acute immobilization tests all parameters (O_2 content and pH) were within the protocol requirements. After 48h, the EC_{50} (Table 1) for *D. longispina* (647.31 mg/L) was almost half of that determined for *D. magna* (1293.05 mg/L). Moreover, the Microtox® test declared that *V. fischeri* was also affected by ASA [EC_{50} for ASA test solutions of 1000 and 1800 mg/L were, respectively, 878.64 (CL: 148.227 to 5208.273 mg/L) and 445.48 mg/L (CL: 397.301 to 499.495 mg/L)].

Table 1

EC_{50} values (mg/L) at 48h exposure with confidence limits (95% probability) in brackets for *D. magna* and *D. longispina* (n = 20).

EC_{50} - 48h	
D. magna	*D. longispina*
1293.05	647.31
(635.599 - 2630.566)	(155.661 - 3694.086)

2. Chronic Tests

According to Fischer's Exact Test, mortalities recorded during chronic exposures in the several treatments were not statistically different from the controls ($P<0.05$). Hence, at 3.20, 5.60 mg/L and 10.00 mg/L, *D. magna* attained the highest mortality percentages (20, 30 and 40%, respectively). Table 2 presents the NOEC and LOEC values obtained to sublethal endpoints related with the female reproductive output and growth.

Table 2

NOECs and LOECs determined for sublethal endpoints analysed for *D. magna* and *D. longispina*.

Endpoint	NOEC (mg/L)		LOEC (mg/L)	
	D. magna	*D. longispina*	*D. magna*	*D. longispina*
Nº viable offspring	1.00	1.00	1.80	1.80
Size of neonates - 1st brood	1.00	>10.00	1.80	>10.00
Neonates per brood				
B1	>10.00	[a]	>10.00	[a]
B2	[a]	1.80	[a]	3.20
B3	1.00	[a]	1.80	[a]
B4	>10.00	[a]	>10.00	[a]
B5	[b]	>10.00	[b]	>10.00
B6	[b]	[a]	[b]	[a]
Age at each reproduction				
B1	>10.00	>10.00	>10.00	>10.00
B2	>10.00	[a]	>10.00	[a]
B3	>10.00	[a]	>10.00	[a]
B4	>10.00	[a]	>10.00	[a]
B5	[b]	[a]	[b]	[a]
B6	[b]	[a]	[b]	[a]
r	>10.00	[a]	>10.00	[a]
DGR	1.00	[a]	1.80	[a]

[a] Not obtainable. [b] B5 or B6 had not occurred. *r* Population growth rate. DGR Daily growth rate

Reproduction

ASA significantly affected the fecundity (number of neonates produced per female) of *D. magna* ($F_{[5,41]}$ = 2.98, P<0.05) and *D. longispina* ($F_{[5,51]}$ = 2.48, P<0.05) (Fig. 2), being the NOECs and LOECs similar to both species (NOEC = 1.00 mg/L; LOEC = 1.80 mg/L).

The number of broods (B) yielded by *D. magna* were 4 versus 5-6 by *D. longispina*, hence the sixth brood had occurred only in one or two adult females (Fig. 3). The size of broods tended to increase all along the test period. In general, there is a reduction on the reproductive output of females subjected to increasing concentrations. In fact, statistical differences between the control and correspondent treatments were recorded in broods B2 ($F_{[5,41]}$ = 3.94, P<0.05) and B3 ($F_{[5,41]}$ = 3.82, P<0.05) for *D. magna*; and B1 ($H_{(0.05,5)}$ = 18.26, P<0.05), B2 ($F_{[5,51]}$ = 5.03, P<0.05), B3 ($F_{[5,51]}$ = 3.16, P<0.05) and B4 ($F_{[5,41]}$ = 5.08,

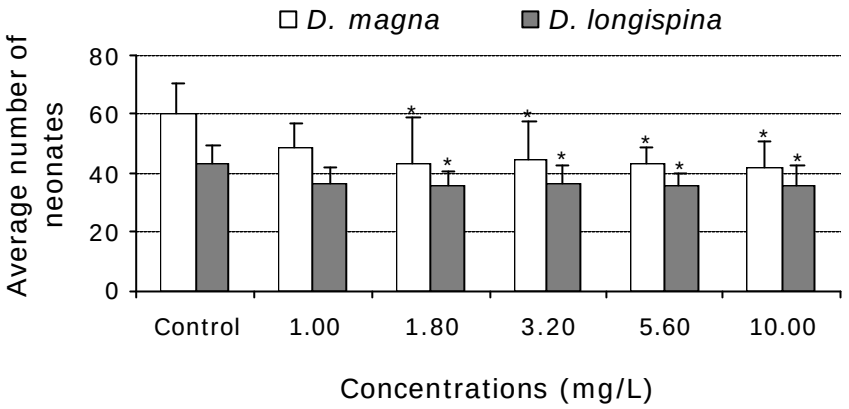

Fig. 2. Number of offspring produced during 21 days by *D. magna* and *D. longispina*, at different concentrations of ASA. Error bars represent the standard deviation, and * indicates a significant value from the control (P<0.05).

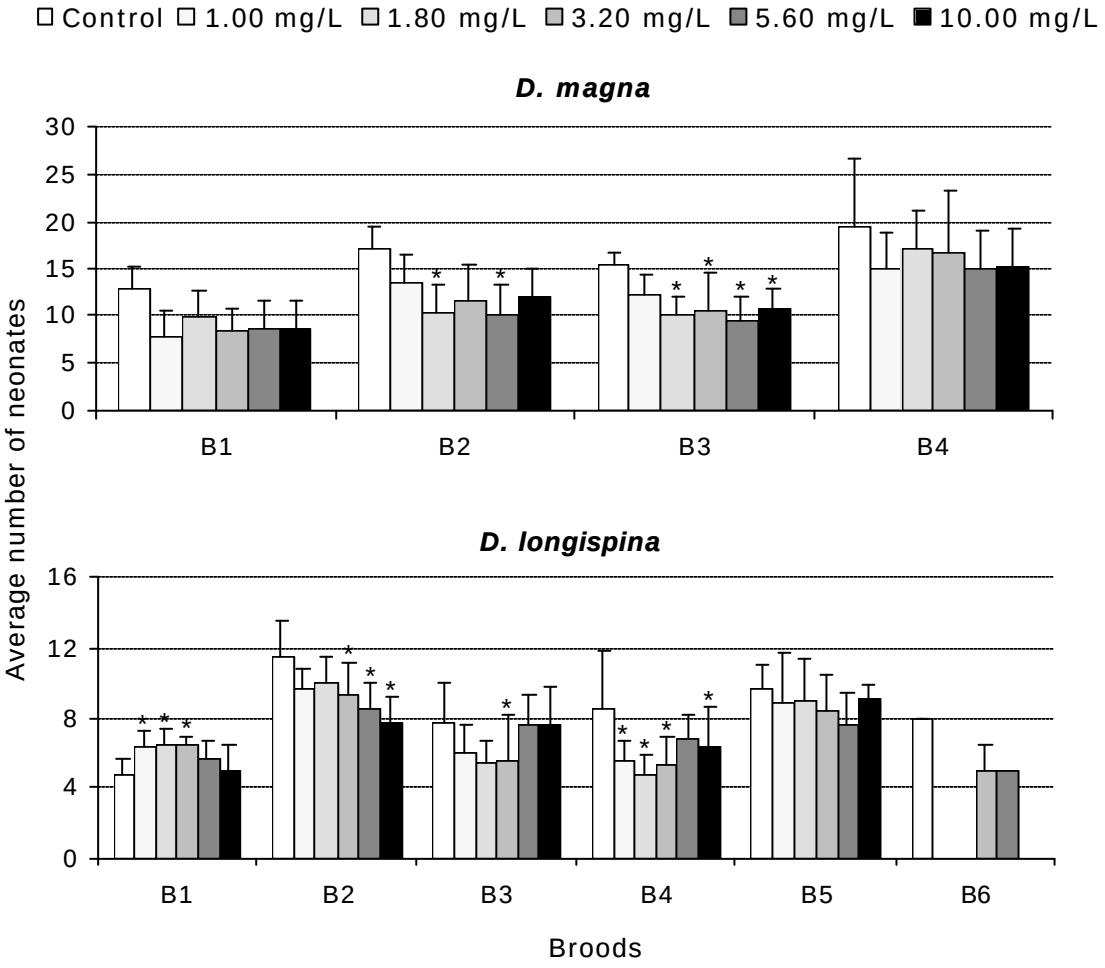

Fig. 3. Number of neonates at each brood (B) produced by *D. magna* and *D. longispina*, at different concentrations of ASA. Error bars represent standard deviation and * indicates a significant value from the control (P<0.05).

P<0.05) for *D. longispina*. However, a statistically significant estimulation of the reproductive output occurred for *D. longispina* in B1 (for the three lower concentrations of 1.00, 1.80 and 3.20 mg/L).

Average age of females at each reproduction is represented in figure 4 for both species. Although no significant differences were observed in *D. magna* between the control and treatments in each brood, there is a slight evidence indicating that females exposed to ASA concentrations released their offspring earlier. The same pattern is shown by *D. longispina*, however, in its case, statistical differences were assessed to the extreme concentrations.

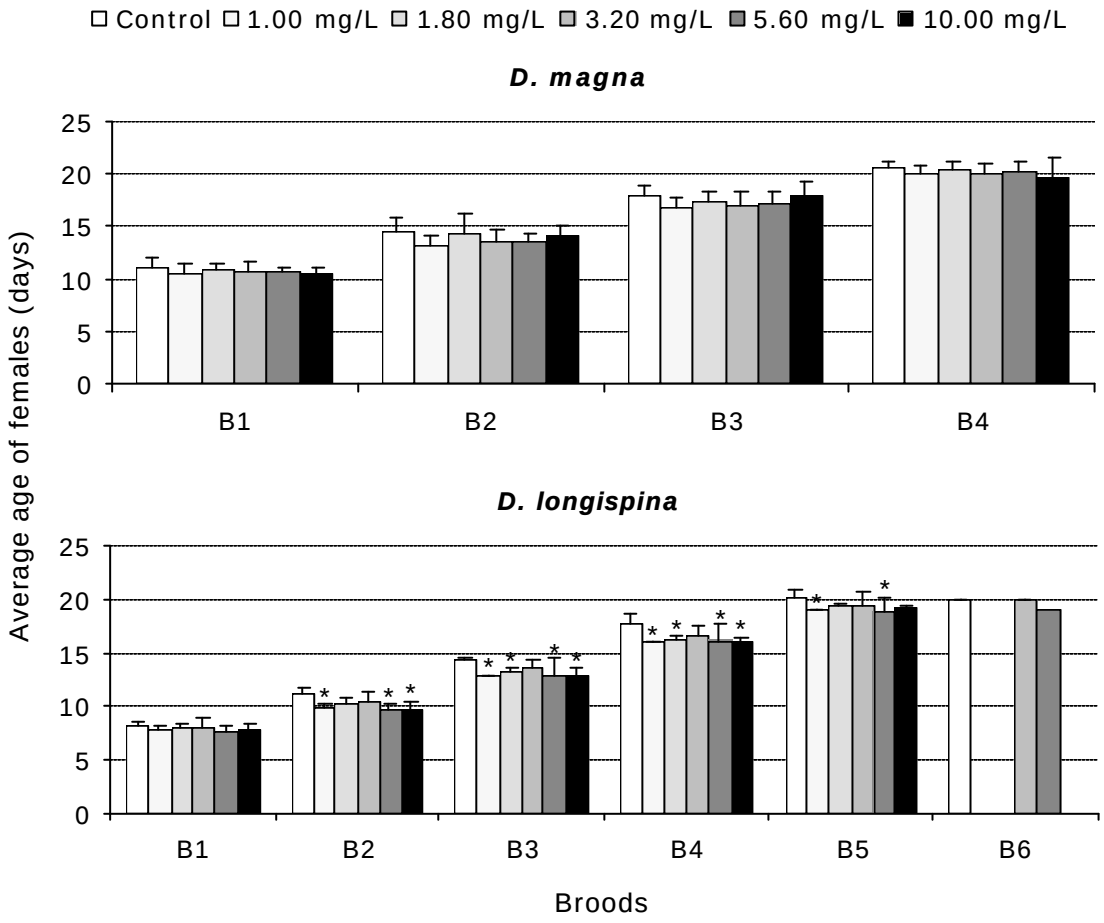

Fig. 4. Average age of females at each brood (B) produced by *D. magna* and *D. longispina*, at different concentrations of ASA. Error bars represent the standard deviation, and * indicates a significant value from the control (P<0.05).

Particularly, the age at first reproduction did not vary in relation to the control and within treatments for *D. magna* (11.1?0.991 to 10.6?0.886 days) and for *D. longispina* (8.2?0.422 to 7.5?0.707 days). In spite of this, differences were recorded concerning the number of neonates produced per female and their size at the first reproduction (Fig. 5). In general, the size of neonates is inversely proportional to the clutch size. This is specially evident for *D. longispina*. Despite the significant estimulation of the production of neonates in the three lowest concentrations, as it was aforementionated, no statistical differences were determined for the size of neonates ($H_{(0.05,5)}$ = 3.27, P>0.05). On the contrary, the size of *D. magna* juvenils was significantly reduced ($H_{(0.05,5)}$ = 27.90, P<0.05), being the NOEC and LOEC values similar to those determined for the fecundity of parent animals (1.00 and 1.80 mg/L, respectively).

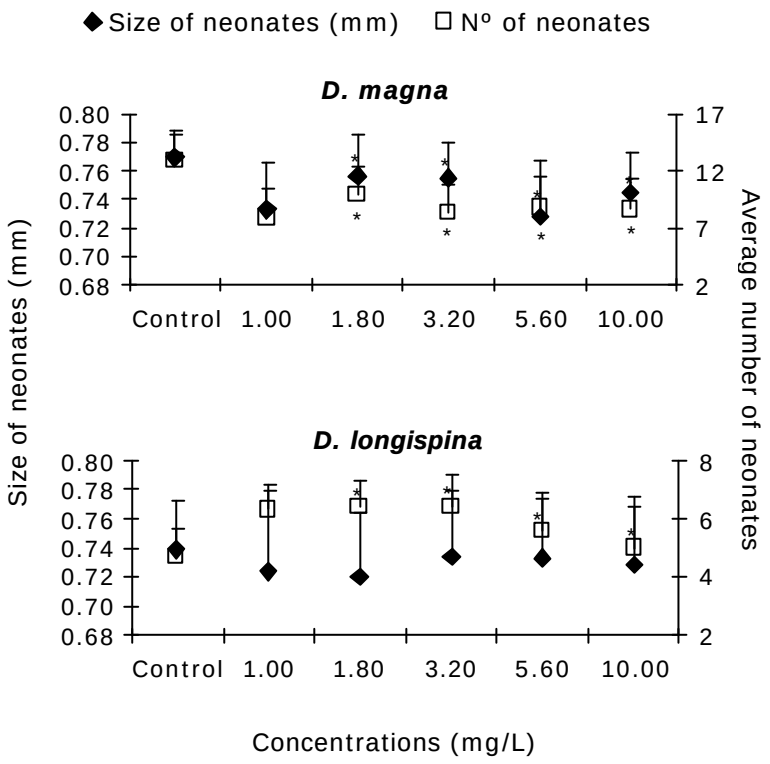

Fig. 5. Average number of neonates from the first brood *versus* their size for *D. magna* and *D. longispina*, at different concentrations of ASA. Error bars represent the standard deviation, and * indicates a significant value from the control (P<0.05).

Growth

The population intrinsic growth rate (*r*) combines both cumulative fecundity and survivorship of individuals during the test period. As it can be verified in figure 6, the *r* of *D. longispina* is greater than that of *D. magna*. Furthermore, increasing concentrations of ASA tend to raise the population growth of *D. longispina*, while the opposite is observed for *D. magna*.

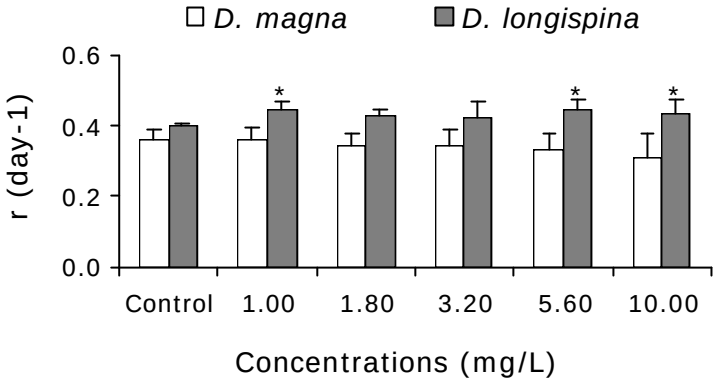

Fig. 6. Population growth rate (*r*) of *D. magna* and *D. longispina*, at different concentrations of ASA. Error bars represent the standard deviation, and * indicates a significant value from the control (P<0.05).

Neverthless, ASA had not significantly affected the *r* of this species ($F_{[50,540]}$ = 2.09, P>0.05; LOEC > 10.00 mg/L), which was unlike for *D. longispina*, as there was a significant induction of its *r* ($F_{[50,540]}$ = 3.68, P<0.05) for some concentrations (1.00, 5.60 and 10.00 mg/L).

Statistical differences were obtained for the DGR of *D. magna* ($F_{[50,410]}$ = 2.87, P<0.05) and *D. longispina* ($H_{(0.05,5)}$ = 11.56, P<0.05), between treatments and control averages (Fig. 7). Moreover, the significant decrease in DGR along the tested concentrations of ASA, had a LOEC = 1.80 mg/L for *D. magna*, while it was impossible to determine NOEC and LOEC values for *D. longispina*.

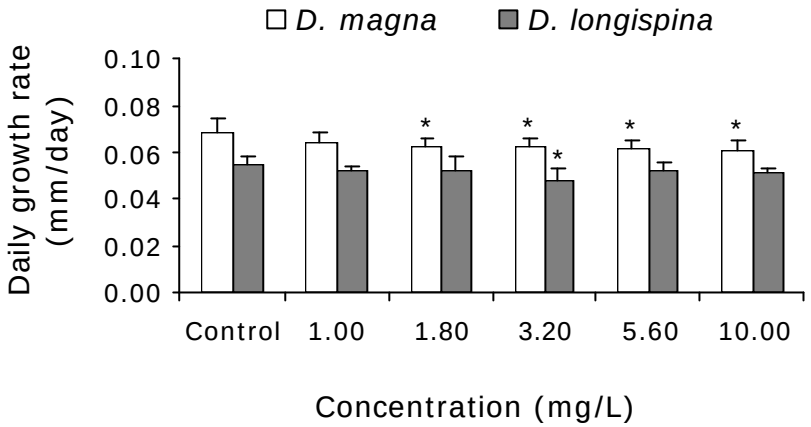

Fig. 7. Daily growth rate (DGR) of *D. magna* and *D. longispina*, at different concentrations of ASA. Error bars represent the standard deviation, and * indicates a significant value from the control ($P<0.05$).

DISCUSSION

The results obtained in our study show that ASA induces acute and chronic toxicity in the test organisms, although the effect concentrations found were far above the ng/L-?g/L concentration range determined in different aquatic samples.

D. longispina, the autochthonous species, was more sensitive to the acute exposures of ASA (EC_{50} = 647.31 mg/L) than the standard one, *D. magna* (EC_{50} = 1293.05 mg/L). Neverthless, the EC_{50} determined for *D. magna* was close to that obtained by Lilius *et al*. (1995), 1468.63 mg/L, despite the lower EC_{50} value calculated in an ealier study conducted by Calleja *et al*. (1993 *in* Lilius *et al.*, 1995), 167.54 mg/L. Moreover, *D. pulex* presented an acute EC_{50} of 360.30 mg/L (Lilius *et al*., 1995), which is near that obtained in this study for *D. longispina*. Other experiments in which rainbow trout hepatocytes and gill epithelial cells were exposed to ASA, indicated acute EC_{50} values of 4972.14 mg/L (Lilius *et al*., 1994) and 852.11 mg/L (Lilius *et al*., 1995), respectively, thereby suggesting the ability of ASA to induce toxic effects in higher trophic levels.

The correlation between *V. fischeri* bacterium and *D. magna* was already proved to be a significant one, which means that it can be exploited to make

reliable predictions from one endpoint to another (Kaiser, 1998). This author pointed out that this works particularly well for compounds of relatively simple chemical structure with one reactive or functional group. In this way, despite the single reactive carboxilic group of ASA, the effect concentrations determined in the Microtox® acute test (EC_{50}s for the concentrations of 1000 and 1800 mg/L of ASA were, respectively, 878.64 and 445.48 mg/L) had shown that not only *V. fischeri* was affected by ASA exposures, but also that they were proximate to those EC_{50}s obtained for *Daphnia* sp.

Most available toxicity data refers to acute effects of antibiotics. Notwithstanding, the continual input of other pharmaceuticals, though present in low levels, unlikely producing acute toxic effetcs, do not take away the possibility that chronic effects may become apparent after many years (Jones *et al*., 2001). Therefore, a life cycle test was carried out with *Daphnia* sp.. During the chronic assay, the mortalities recorded were higher for the standard species when exposed to the three highest concentrations of ASA, contributing to the quite elevated standard deviations of some parameters determined. Thus, the number of replicates should be incresead in future toxicity tests performed with *Daphnia* sp., as a possible solution to reduce the standard deviations.

Relativelly to the chronic assay, the LOEC determined for the reproductive output of both species was 1.80 mg/L, which is much lower than the chronic EC_{50} value of 61-68 mg/L reported by Stuer-Lauridsen *et al.* (2000) for *D. magna*. Indeed, there was a decreasing trend on fecundity for both daphnid species in each released brood, when exposed to increasing concentrations of ASA. However, statistical analysis revealed a significant inhibition only in intermediate broods (B2 and B3 for *D. magna*, B2-B4 for *D. longispina*), reflecting the sensitiviness of females under longer exposures to ASA. Overall, in the first brood, a significant low-dose stimulation of the production of neonates by *D. longispina* had occurred in opposition to a high-dose inhibition. This fact could be related to an hormetic response, which amplitude is limited to no more than a factor of two above the control value (Calabrese, 2002). In other studies, though no precise explanation could be given, it was detected a statistically significant stimulation of the reproductive output of *D. magna* at some test concentrations of three antibiotics (Oxolinic acid, Sulfadiazine, Tylosin) (Wollenberger *et al*., 2000); whereas, Migliore *et al*. (2000) reported hormesis in the development of

the aquatic weed *Lythrum salicaria* L., after a 35-day exposure to lower concentrations (50-5000 ?g/L) of the antibiotic flumequine.

Life history traits normally analysed in reproduction tests give special attention to the first brood due to its ecological relevance, as its release minimizes the extinction of the species in ecosystems, whenever their maintenance is constrained (Stibor and Lampert, 1993). When comparing the number and size of neonates released at first reproduction, some differences were recorded. Firstly, a slight negative correlation is apparent between the two variables, specially for *D. longispina*. Several studies reported that under stress conditions (*e.g.*, low quantity and quality of food available), daphnids tend to produce less eggs and neonates, with bigger size (Stross and Bernardi, 1997; Abrantes, 2002). Secondly, in spite of this, a significant decreasing trend of *D. magna* neonates size (LOEC = 1.80 mg/L) occurred to increasing levels of ASA, which means that ASA was somehow affecting the daphnids first reproduction offspring. In order to achieve a better understanding on this issue it would have been interesting to evaluate the mass and size of neonates from sucessive clutches.

In that *r* explicity integrates individual-level traits – *i.e.*, survival, reproductive output and period between sucessive reproductions - to toxicants at the population level, it is, by definition, a better ecotoxicological endpoint providing a more relevant measure of ecological impact (Forbes and Calow, 1999). Increasing concentrations of ASA diminished *D. magna* population growth (though not significantly), which is in close agreement with its mortality and fecundity rates recorded during the test. In opposition, the *r* of *D. longispina* had been significantly enhanced, in spite of the decreasing trend of its reproductive output. Moreover, the pattern of the population growth of *D. magna* was not only inverse to that observed for *D. longispina*, but it also assumed lower values. This evidence could be related with the total number of broods produced per each species, as well as the female age at each reproduction. As a matter of fact, the autochthonous cladoceran, *D. longispina*, had lower mortality rates and produced more broods than the standard species. In addition, mature females of *D. longispina* exposed to ASA, released their offspring significantly earlier (from the control) for the extreme concentrations of toxic, which is in accordance with the determined significant values of *r*. *D. magna*, although, presented the same

pattern relatively to females age at each brood release, no statistical differences were attained. Thus, this different trial trends may contribute to the different autochthonous and standard cladocerans population growth observed.

Furthermore, Forbes and Calow (1999) explained that life-table analysis can provide important insights into the mechanisms of population-level consequences of toxicant exposure, and can be used to generate testable hypotheses to explain why certain species dominate in polluted areas, whereas others disappear at the earliest stages or lowest degrees of toxicant exposure. Considering that *D. longispina* is an autochthonous species, which means that, apparently, it has a developed ability to adjust its life trait strategies under adverse conditions, it is more likely that it maintains or enhances its fitness than *D. magna*, thereby explaining the aforementionated increasing *r* for *D. longispina* under ASA exposures. The *r* determined for both species controls was in accordance with those calculated for *D. magna* (Forbes and Calow, 1999; Trubetskova and Lampert, 2002), *D. pulex* (Meyer *et al.*, 1987 *in* Forbes and Calow, 1999), *D. parvula* and *D. ambigua* (Winner and Farrell, 1976 *in* Forbes and Calow, 1999).

The daily growth rate was also affected by ASA. While for *D. magna*, the DGR was significantly inhibited at the LOEC of 1.80 mg/L, for *D. longispina*, it had a slight reduction.

Thus, in general, chronic exposures to ASA induced a decrease in normal reproduction and growth of both standard and autochthonous daphnids. According to Trubetskova and Lampert (2002), this is a possible toxic effect on *Daphnia*, which can occur through a reducing trend in energy supply, probably through feeding (*e.g.*, reduced ingestion or assimilation rates) (*i.e.*, "supply-side responses"), and an implied increase in maintenance (*i.e.*, "demand-side responses") *sensu* Baird *et al.* (1990). However, another explanation reported by Hanazato (1998; 2001), postulated that the number of offspring per clutch is not affected directly by toxic chemicals. Rather it is a function of maternal body size, which in turn is governed by growth rate during the juvenile stage. Therefore, the decreasing pattern of the females DGR herein observed for increasing concentrations of ASA, probably means that the females growth was perturbed at levels resulting in the inhibition of reproduction.

Comparing and summarizing the responses of *D. magna* and *D. longispina* it is quite clear that the former was more tolerant to acute ASA exposures, while

the latter, generally, seemed to be more resistant, mainly at the population-level traits, and could better withstand the harmful circumstances under long term exposures.

Regardless the high effect concentrations obtained in this study, pharmacological compounds detected in the aquatic environment occur at much lower levels. In spite of this, one cannot put away the possibility that cumulative, imperceptible effects may be induced on non-target individuals when continuously exposed to them in their natural environment. As a matter of fact, *Daphnia* sp. is provided with receptor systems similar to those of vertebrate species (Barry and Stoopman, 2000), over which pharmaceuticals are specifically designed to act on. Because of this, pharmacological residues have, *a priori*, the potential to exert changes upon physiological and/or morphological functions of daphnids, which effects can be ultimately manifestated after long periods of chronic exposure that overcome the considered test period. Thus, it would be interesting to assess the effect of pharmacological compounds at a lower organizational level (*e.g.*, biochemical level), in order to detect first warning responses to changes in normal living conditions.

CONCLUSION

The pharmacological effects of ASA are likely to impare the survivorship, reproduction and growth of cladoceran species, as well as, the reduction of the bioluminescence of *V. fischeri*. However, the required concentrations to induce these effects within the test period are extremely higher in comparison to that determined in the aquatic environment.

While *D. magna* was the most tolerant species when subjected to acute levels of ASA, *D. longispina* showed to be more resistant and able to withstand its population dynamics under long term unfavourable toxic conditions. Therefore, autochthonous species seem to give a more feasible and ecological relevant understanding about their behaviour under chronic environmental agressions, in particular, those consisting of constant input of pharmaceuticals.

Actually, environmental exposures to pharmacological compounds occur at very low levels. Neverthless, due to their specific biological active natures, pharmaceuticals have the potential to exert very subtle physiologic and/or morphologic effects on non-target organisms, which may escape to detection during the testing period, but slowly they can accumulate over time, resulting at an ultimate level, in substantive outward changes.

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Capítulo III

Life-History Traits of Standard and Autochthonous Cladocerans:
II. Acute and Chronic Effects of Acetylsalicylic Acid Metabolites

Life-History Traits of Standard and Autochthonous Cladocerans: II. Acute and Chronic Effects of Acetylsalicylic Acid Metabolites

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ABSTRACT

Metabolic products are often more toxic than their pharmacological parent compounds. Therefore, acute and chronic effects of acetilsalicylic acid (ASA; active ingredient of Aspirin® and many other pharmaceuticals) main metabolites (salicylic acid, SAL; gentisic acid, GEN; *o*-hydroxyhippuric acid, HDP) were assessed on standard (*Daphnia magna*) and autochthonous (*Daphnia longispina*) cladocerans. The bioluminescence inhibition was also determined for *Vibrio fischeri*. The bioluminescence intensity was inhibited only by SAL at the highest test solution concentration (EC_{50} = 669.46 mg/L; confidence limit: 464.875 to 964.079 mg/L). The sequence of acute and chronic decreasing toxicity of ASA metabolites for daphnids was GEN > SAL > HDP. Acute toxicity of HDP was unlike but chronic exposures enabled the production of abnormal neonates and, particularly, egg abortion. Thus, reproduction was the most susceptible endpoint to HDP. On the other hand, SAL and GEN induced changes on normal patterns of reproduction and growth for both species. In general, *D. longispina* was more sensitive than *D. magna*, although the autochthonous species could better withstand their population growth under SAL exposures than the standard one. Overall, the determined effect concentrations were above the levels detected in aquatic environmental samples. Neverthless, long term exposures to low levels of pharmacological active substances, usually present as complex mixtures, may induce slight physiological and biochemical changes in non-target organisms, that can cumulate over time, and thus manifestate delayed effects on individual- and even population-level traits.

Key words : acetylsalicylic acid, metabolites, toxicity, *Daphnia magna*, *Daphnia longispina*, *Vibrio fischeri*.

INTRODUCTION

Drug residues have become a noteworthy contamination factor in a wide range of aquatic environmental samples (Richardson and Bowron, 1985; Halling-Sørensen *et al.*, 1998; Ahrer *et al.*, 2001, Heberer *et al.*, 2002), at concentrations that rival to those of some pesticides (Jones *et al.*, 2001). In spite of this, data regarding the toxicity of pharmaceutically active compounds (PhACs) are quite rare, specially those referring to their metabolites (Ternes, 1998; Stumpf *et al.*, 1999).

Acetylsalicylic acid (ASA; 2-(acetyloxy)benzoic acid), which is the active compound of Aspirin® and an ingredient of several other pharmaceuticals, is an over-the-counter analgesic worldwide consumed in great amounts (Heberer, 2002a). Following oral administration, part of ASA is excreted without changes, being the remainder rapidly hydrolysed to salicylic acid (SAL; 2-hydroxybenzoic acid) in the intestinal wall, liver and red blood cells. This is the main metabolite, although it is further metabolized by glycine conjugation to *o*-hydroxyhippuric acid (HDP; also known as salicyluric acid; *N*-(2-hydroxybenzoyl)glycine) and by hydroxilation to gentisic acid (GEN; 2,5-dihydroxybenzoic acid) (Patel *et al.*, 1990; DeBlassio *et al.*, 2000; Zaugg *et al.*, 2001) (Fig. 1).

SAL is considered to be the active metabolite responsible for most of the therapeutic effects of ASA and it is the first excreted metabolite entering waste water (Olguín *et al.*, 2001). Neverthless, residues of SAL do not necessarily have to derive from ASA. Other sources such as the use of SAL as keratolytic, dermatice, and preservative of food or its natural formation are even more likely to be responsible for the occurrence of this compound in the aquatic environment (Heberer, 2002b).

Actually, in German sewage influents were detected maximum concentration levels ranging from 0.34 ?g/L (Heberer, 2002b) to 54 ?g/L for SAL; >6.8 ?g/L for HDP; and >4.6 ?g/L for GEN (Ternes *et al.*, 1998). Although sewage treatment works (STWs) are quite efficient on the removal of this micropollutants, Ternes *et al.* (1998) reported the presence of SAL (0.14 ?g/L) and GEN (0.59 ?g/L) in STWs discharges, while other authors (*e.g.*, Hignite and Azarnoff, 1977; Heberer *et al.*, 2001; Farré *et al.*, 2001; Flaherty *et al.*, 2002; Heberer, 2002b) detected residues of SAL at concentrations ranging 0.04-95.6

?g/L. Maximum loading levels of SAL and GEN measured in surface water samples were 4.1 ?g/L and 1.2 ?g/L (Ternes, 1998), respectively.

Besides the low levels and the reduced half-lives of PhACs in the aquatic environment, the continuous introduction of drugs by sewage effluent makes them “pseudo-persistent” pollutants with implications for the aquatic organisms (Daughton, 2002). Some authors (*e.g.*, Lilius *et al.*, 1995; Wollenberger *et al.*, 2000) have assessed acute effects of PhACs on aquatic test organisms. However, it is still unclear the chance of occurring cumulative subtle changes during long-term exposures of non-target populations, that would otherwise be attributed to natural adaptations.

Therefore, the present work intends to assess the toxic effects of ASA metabolites (SAL, HDP and GEN) on acute and sub-lethal responses of two cladocerans: *Daphnia magna* Straus, a standardized test organism often used on aquatic toxicology, and *Daphnia longispina* O.F. Müller, an autochthonous species widely distributed in Portuguese shallow lakes (Barros, 1994; Pereira, 1997; Abrantes, 2002). Additionally, it was also determined the acute toxicity of SAL, HDP and GEN on a bioluminescent bacterium - *Vibrio fischeri* (Beijerinck) Lehmann et Neumann - used to perform the Microtox? test.

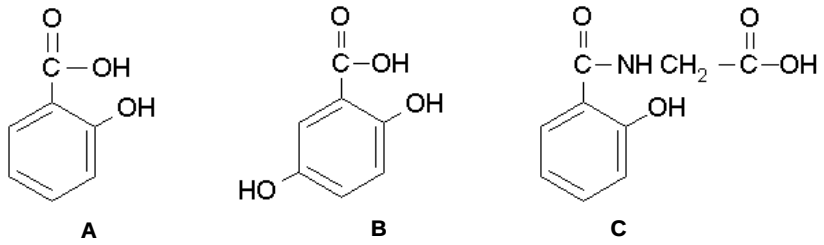

Fig. 1. Chemical structure of SAL (A), GEN (B) and HDP (C).

MATERIAL AND METHODS

Daphnids Culture Method

Population cultures of daphnids were maintained in 1 L glass beakers filled with 800 ml of ASTM hard water (ASTM, 1980; EPA, 1989), hereinafter referred to as ASTM. An organic additive made of *Ascophylum nodosum* (L.) Le Joli seaweed extract (Baird *et al.*, 1988) prepared by dilution of a stock solution (Soares, 1989) was added to the culture medium at a concentration of 6 ml/L. Individuals were fed every two days with *Pseudokirchneriella subcapitata* (Korshikov) cultured in the lab (Stein, 1973) (3.00×10^5 and 1.50×10^5 cell/ml/*Daphnia* for *D. magna* and *D. longispina*, respectively). Medium renewal was undertaken three times per week. The cultures were kept with no aeration supply, at a 16^L:8^D photoperiod and a temperature of 20 ? 1ºC. The used clones of daphnids were acclimated to lab environmental conditions for at least ten generations in order to minimize the environmental variation effects experienced by their ancestrals, according to Soares (1989).

Chemicals and Test Solutions

Chemicals were obtained from Sigma-Aldrich Chemie?, Germany (SAL: 99% pure, GEN: 98% pure, HDP: 100% pure). Test solutions of SAL, GEN and HDP were made up by dilution of a stock solution, which was prepared with ASTM previously at the beginning of the test. The pH of the stock solutions was adjusted to the 6-9 range. During the experimental chronic period the stock solutions were stored at 2-8 ºC in dark. The nominal concentrations used on SAL, GEN and HDP acute immobilization tests were within the range of 900.00-2350.00 mg/L, 180.00-910.00 mg/L and 180.00-1800.00 mg/L, respectively; whereas reproduction tests were conducted for 1.00, 1.80, 3.20, 5.60 and 10.00 mg/L of SAL; 0.32, 0.56, 1.00, 1.80 and 3.20 mg/L of GEN; 38.4, 84.5, 186.0, 409.0 and 900.0 mg/L of HDP, for both daphnids species. The concentrations tested in Microtox? were between 180.00-1800.00 mg/L for SAL and HDP, and 56.00-560.00 mg/L for GEN compound.

Experimental Design

1. Tests with Daphnids

Tests were conducted according to OECD guidelines for *Daphnia sp.* Acute Immobilization Test (OECD, 2000), and *D. magna* Reproduction Test (OECD, 1996), being the last one adapted to *D. longispina*. All experiments were carried out with a single clone of *D. magna* (clone A *sensu* Baird *et al.*, 1989) and *D. longispina* (EM7 clone *sensu* Antunes *et al.*, in press). In order to minimise maternal effects, only neonates from the third to fifth broods (Barata and Baird, 1998) ageing less than 24h were tested.

Immobilization Test. Daphnids were maintained in 180 ml glass vessels for 48h, with 50 ml of ASTM and test solutions. Four replicates of five individuals each were used per treatment, including the ASTM control. Incubation conditions were the same as those described for the culture procedure with the exception that animals were not fed. The dissolved oxygen (oxi 330 WTW) and pH (pH 330 WTW) were measured at the beginning and at the end of the test. After 24 and 48h the results were recorded, although it will only be presented the EC_{50} at 48h. The criterium for toxic effect was immobilization of the neonates upon beaker swirling stimulation.

Reproduction Test. Daphnids used in 21-day-chronic assay were individually pooled in 50 ml glass vessels filled with ASTM and test solutions in appropriate volumes. Ten replicates were performed for the control and for each tested concentration. Experimental conditions were similar to those already described above for the culture regime, including the feeding and renewal procedure. Oxygen concentration and pH levels in vessels were measured weekly to guarantee that they were not limitting factors of biologic responses. During the test, the recorded and measured parameters were mortality, total number of neonates, number and size of broods, average size of three neonates randomly chosen from the first brood, females age at each brood release, length (Olympus SZX9 esteromycroscope with an ocular micrometer) of the first exopodite of the second antennae of the first moult, and the last molt released within 21 days. The conversion of antennae length (AL) in body length (BL) was achieved through the allometric relations (1) and (2) for *D. magna* and *D. longispina*, respectively. Regression (1) was obtained by measuring the AL and BL of 416 *D.*

magna individuals during their life cycle (unpublished data), while equation (2) was determined by Silva (1999).

$$BL_{D.magna} = 10.558 \times AL_{D.magna} - 0.3475 \ (mm) \ (r^2 = 0.9615) \quad (1)$$

$$BL_{D.longispina} = 10.5 \times AL_{D.longispina} - 0.1437 \ (mm) \ (r^2 = 0.9621) \quad (2)$$

To assess the effects on normal growth it was determined the daily growth rate (DGR), according to equation (3):

$$DGR = [\ln(BL_f) - \ln(BL_i)] \ / \ ?t \quad (days^{-1}) \quad (3)$$

where, BL_f is the organism final body length (mm) after 21 days, BL_i is its initial body length (mm) and $?t$ is the time interval (days) (Sobral, 1997; Burns, 2000). Furthermore, it was also calculated the rate of increase of the population (*r*, day^{-1}) by means of the Euler-Lotka equation (4):

$$? \ e^{-rx}.l_x.m_x = 1 \quad (4)$$

where, x is the age class (days; 0...n), l_x is the probability of surviving at age x, and m_x the fecundity at age x. Standard deviation was determined according to Jackknife technique (Meyer *et al.*, 1986).

2. Microtox? Test

The Microtox? test (Microbics Corporation Inc. Protocols, 1988) is based on the inhibition of the luminescence of the marine bacterium *Vibrio fischeri* after 30 minutes of exposure to the toxicant (Kaiser, 1998; Froehner *et al.*, 2000). In this case it was conducted a Basic Test Protocol (Microbics M500 Toxicity Analyzer), in which *V. fischeri* was subjected to several dilutions of the acute concentrations of SAL, GEN and HDP.

Data Analysis

From each acute immobilization test, the number of immobilized organisms was plotted against the concentrations tested, and a 48h EC_{50} was calculated by the standard probit procedure (Finney, 1971).

Mortalities that occurred during the chronic exposure were analysed by Fischer's Exact Test (EPA, 1989). No-observed effect concentrations (NOEC) and lowest-observed-effect concentrations (LOEC) for reproduction and growth were attained using one-way ANOVA, followed by a Dunnett's test for multiple comparisons of the individual concentration effect and that of controls (Zar, 1996). Square and logarithimic transformations were conducted whenever normality and/or homocedasticity failed. A Kruskal-Wallis test followed by the Dunn's test (testing concentrations *versus* control) was performed if assumptions of parametric ANOVA were still not met. A statiscally significant difference in reproduction or growth is reported for $P<0.05$. Parent animals which died before the end of the test, including their offsprings, were excluded from the statistical analysis.

Microtox® EC_{50} results together with its 95% confidence limits (CL) were determined by the Microtox Software – Microbics Corporation MTX.EXE Version 6.01.

RESULTS

1. Acute Tests

During the acute assay, the variation of pH and oxygen content in the test vessels was in accordance with the guidelines (OECD, 2000), and the percentage of immobilized daphnids in the control was not more than 10%. The EC_{50}s obtained for the 48h acute immobilization test, for the three toxics are described in table 1. HDP was not toxic for both cladocerans and for the bacterium species, as the EC_{50}s were always above the tested concentrations. On the contrary, GEN had the least EC_{50} values in the acute immobilization test, therefore representing a high toxicity for cladocerans species, specially for *D. longispina* (EC_{50} = 342.41

mg/L). No effect was observed in *V. fischeri* bioluminescence when it was subjected to different levels of GEN. SAL seemed to have an intermediate acute toxicity to daphnids, though, *D. longispina* (EC_{50} = 1147.57 mg/L) was, once again, more sensitive than *D. magna* (EC_{50} = 1945.32 mg/L). The highest concentration of SAL (1800 mg/L) induced the reduction of the bioluminescent intensity of *V. fischeri* (EC_{50} = 669.46 mg/L; CL: 464.875 to 964.079 mg/L).

Table 1

EC50 values (mg/L) for *D. magna* and *D. longispina*, after 48h exposures to SAL, GEN and HDP. 95% confidence limits are indicated in brackets (n = 20).

SAL		GEN		HDP	
D. magna	*D. longispina*	*D. magna*	*D. longispina*	*D. magna*	*D. longispina*
1945.32 (1166.189 - 7535.699)	1147.57 (1022.354 - 1279.492)	402.55 (380.902 - 425.402)	342.41 (310.631 - 379.997)	>1800.00	>1800.00

2. Chronic Tests

All the performed reproduction tests with *Daphnia* spp. were valid in accordance with the protocol, as the mean number of offspring in the controls was ? 60 (except for *D. longispina*) with a variation coefficient of less than 25%. Control mortalities did not exceed 20% at the end of the test period.

Mortalities observed during the chronic assay were not significant according to Fischer's Exact Test, although high mortalities were recorded in *D. magna* and *D. longispina* individuals subjected, respectively, to GEN and HDP. The endpoints, then, assessed concerning reproductive and growth parameters, are described as follows in table 2.

Table 2

NOECs and LOECs determined for the several endpoints analysed for *D. magna* and *D. longispina*, exposed to SAL, GEN and HDP.

Endpoint	Treatment	NOEC (mg/L)		LOEC (mg/L)	
		D. magna	*D. longispina*	*D. magna*	*D. longispina*
Nº of viable offspring	SAL	>10.00	5.60	>10.00	10.00
	GEN	0.32	0.32	0.56	0.56
	HDP	186.00	84.50	409.00	186.00
Average size of juvenils from the 1st brood	SAL	*a*	*a*	*a*	*a*
	GEN	>3.20	0.56	>3.20	1.00
	HDP	409.0	84.5	900.0	186.0
Neonates per brood					
B1		>10.00	<1.00	>10.00	<=1.00
B2		>10.00	<1.00	>10.00	<=1.00
B3	SAL	>10.00	>10.00	>10.00	>10.00
B4		*a*	1.80	*a*	3.20
B5		*a*	>10.00	*a*	>10.00
B6		*b*	*a*	*b*	*a*
B1		>3.20	*a*	>3.20	*a*
B2		>3.20	1.00	>3.20	1.80
B3	GEN	<0.32	0.56	<=0.32	1.00
B4		*a*	*a*	*a*	*a*
B5		*a*	1.80	*a*	3.20
B6		*b*	*a*	*b*	*a*
B1		409.0	*c*	900.0	*c*
B2		409.0	*c*	900.0	*c*
B3	HDP	409.0	*c*	900.0	*c*
B4		409.0	*c*	900.0	*c*
B5		*b*	*c*	*b*	*c*
B6		*b*	*c*	*b*	*c*
Age at each reproduction					
B1		>10.00	>10.00	>10.00	>10.00
B2		>10.00	*a*	>10.00	*a*
B3	SAL	>10.00	<1.00	>10.00	<=1.00
B4		>10.00	<1.00	>10.00	<=1.00
B5		>10.00	1.00	>10.00	1.80
B6		*b*	*a*	*b*	*a*
B1		>3.20	>3.20	>3.20	>3.20
B2		>3.20	>3.20	>3.20	>3.20
B3	GEN	>3.20	>3.20	>3.20	>3.20
B4		*a*	0.56	*a*	1.00
B5		>3.20	>3.20	>3.20	>3.20
B6		*b*	*a*	*b*	*a*
B1		>900.0	>900.0	>900.0	>900.0
B2		>900.0	>900.0	>900.0	>900.0
B3	HDP	>900.0	>900.0	>900.0	>900.0
B4		>900.0	>900.0	>900.0	>900.0
B5		*b*	*a*	*b*	*a*
B6		*b*	*b*	*b*	*b*
r	SAL	>10.00	<1.00	>10.00	<=1.00
	GEN	1.80	>3.20	3.20	>3.20
	HDP	*a*	84.5	*a*	186.0
DGR	SAL	5.60	>10.00	10.00	>10.00
	GEN	0.56	1.80	1.00	3.20
	HDP	>900.0	>900.0	>900.0	>900.0

[a] Not obtainable. [b] No brood occurred. [c] Not calculated. *r* Population growth rate. DGR daily growth rate.

Reproduction

Apparently, there was a dose-response relationship between SAL, GEN and HDP concentrations and the number of neonates produced by *D. magna* and *D. longispina* (Fig. 2). Except for *D. magna* subjected to SAL ($F_{[5,47]}$ = 0.87, P>0.05), a significant decrease in the reproductive output was determined for *D. longispina* exposed to SAL ($F_{[5,50]}$ = 3.89, P<0.05), and for both daphnids species exposed to GEN (*D. magna*: $F_{[5,27]}$ = 7.15, P<0.05; *D. longispina*: $H_{(0.05,5)}$ =21.48, P<0.05) and HDP (*D. magna*: $F_{[5,51]}$ = 84.93, P<0.05; *D. longispina*: $H_{(0.05,5)}$ = 49.47, P<0.05).

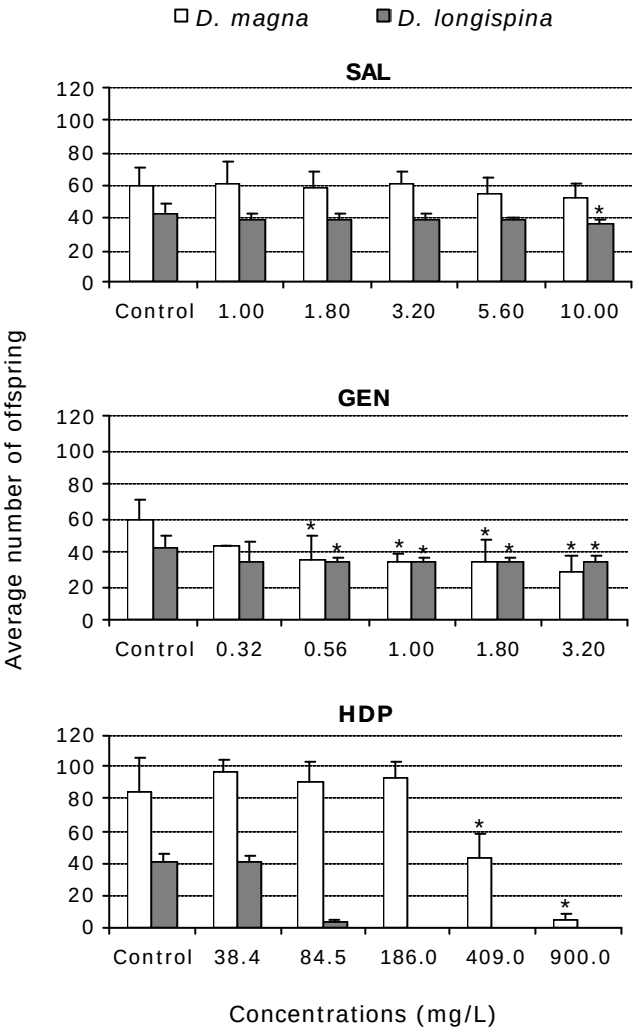

Fig. 2. Number of viable offspring produced during 21 days by *D. magna* and *D. longispina*, at different concentrations of SAL, GEN and HDP. Error bars represent the standard deviation, and * indicates a significant difference from the control (P<0.05).

Moreover, GEN inhibited the reproduction of both species at lower concentrations (LOEC = 0.56 mg/L) than SAL (*D. magna*: LOEC >10.00 mg/L; *D. longispina*: LOEC = 10.00 mg/L) or HDP (*D. magna*: LOEC = 409.0 mg/L; *D. longispina* had suffered a drastic reduction, impossible to present NOEC and LOEC values).

Surprisingly, there was a stimulation, though not significant, of the production of neonates in *D. magna* females subjected to the three lower concentrations of HDP in relation to the control. On the other hand, the drastic decrease of the number of neonates at 84.5 mg/L, for *D. longispina*, and at 186.0 mg/L, for *D. magna*, reflects the increasing number of aborted eggs and undifferenciated neonates meanwhile released at the same time as the moults (Figs. 3A and 3B).

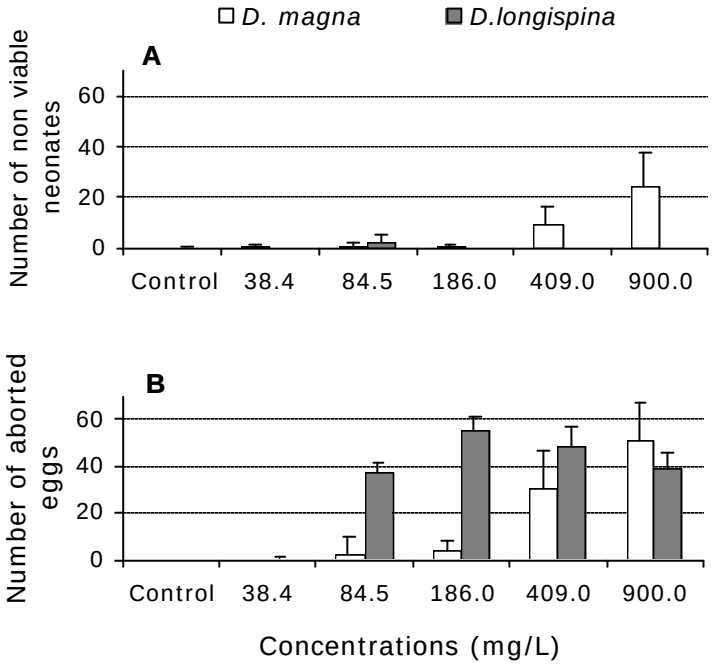

Fig. 3. Number of non viable neonates (A) and aborted eggs (B) produced by *D. magna* and *D. longispina*, at different concentrations of HDP. Error bars represent the standard deviations.

Unviable offspring is mainly represented by the abortion of eggs (Figs. 4A and 4B), although *D. magna* had produced some malformed neonates at 409.0 and 900.0 mg/L of HDP (Fig. 4C). This effect was more pronounced in *D. longispina* and started to occur at lower concentrations than those observed for

D. magna. Neverthless, in the presence of higher concentrations the production of offspring had reduced, as the number of eggs released by the former had diminished, contrary to *D. magna* reproductive behaviour.

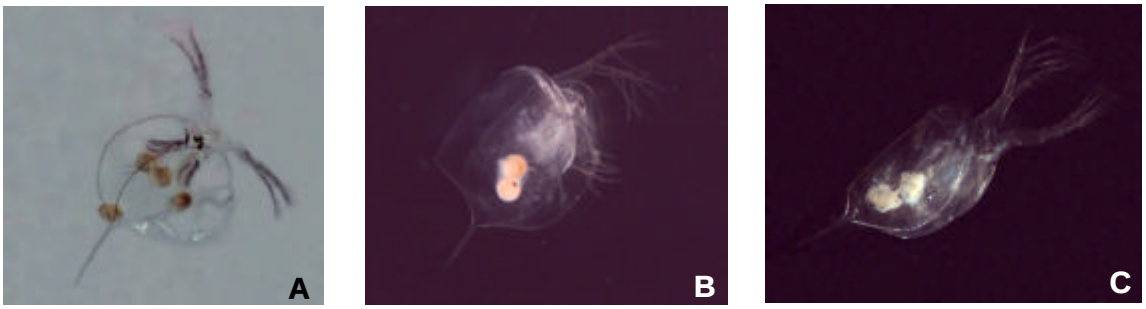

Fig. 4. Moults carrying aborted eggs (A – *D. longispina*, at 186.0 mg/L of HDP, BL = 0.953 mm - and B – *D. magna*, at 900.0 mg/L of HDP, BL = 2.43 mm) and malformed juvenils (C – *D. magna*, at 900.0 mg/L of HDP, BL = 2.07 mm).

The number of neonates produced per clutch (B) tends to raise as females grow older, which is clearly evident in HDP for both daphnid species (Fig. 5). Usually, *D. magna* produces less broods (four-five) than *D. longispina* (five-six). In a general view, the number of released offsprings of *D. magna* and *D. longispina* tends to diminish to increasing SAL, GEN or HDP concentrations.
Actually, the statistical analysis performed revealed significant differences in *D. magna* clutch sizes: B4 for SAL exposures ($F_{[5,47]}$ = 1.37, P<0.05); B3 ($F_{[5,26]}$ = 10.18, P<0.05) and B4 ($F_{[4,15]}$ =4.30, P<0.05) for GEN exposures. *D. longispina*, however, was more affected in its reproductive output during the test period than *D. magna*: significance had been obtained in B1 ($F_{[4,15]}$ = 4.30, P<0.05), B2 ($F_{[4,15]}$ = 4.30, P<0.05) and B4 ($F_{[4,15]}$ = 4.30, P<0.05) for SAL; whereas in the treatment with GEN, B1 ($F_{[4,15]}$ = 4.30, P<0.05), B2 ($F_{[4,15]}$ = 4.30, P<0.05), B3 ($F_{[4,15]}$ = 4.30, P<0.05), B4 ($F_{[4,15]}$ = 4.30, P<0.05) and B5 ($F_{[4,15]}$ = 4.30, P<0.05) had shown differences between the tested concentrations and the control.

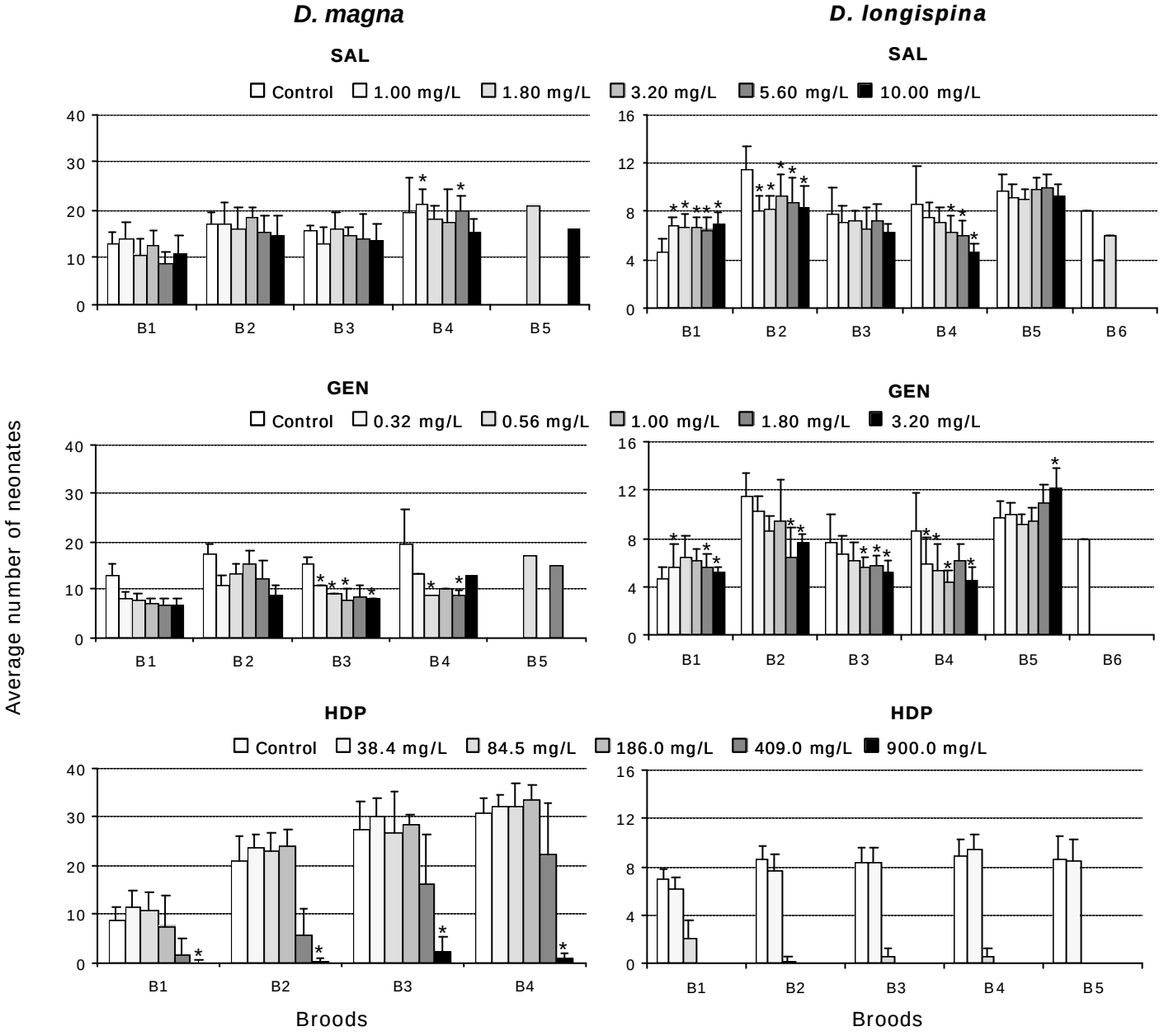

Fig. 5. Number of viable neonates per brood (B) produced by *D. magna* and *D. longispina*, at different concentrations of SAL, GEN and HDP. Error bars represent the standard deviation, and * indicates a significant difference from the control ($P<0.05$).

Nevertheless, there are some exceptions to the decreasing pattern of the clutch size as the concentration increases. HDP exposure induced an increase of the number of offspring of *D. magna* to lower concentrations in almost every clutch, as it was already pointed out to the total number of neonates, though again not significant. In addition, the reproductive output of *D. longispina* is significantly enhanced in B1 to all concentrations of SAL (NOEC < 1.00 mg/L), as

well as in the GEN treatment for B1 (for concentrations of 0.56 and 1.00 mg/L) and B5 (LOEC = 3.20 mg/L).

The age of parent daphnids at each produced brood tends to decrease when they are exposed to the three toxic compounds (Fig. 6).

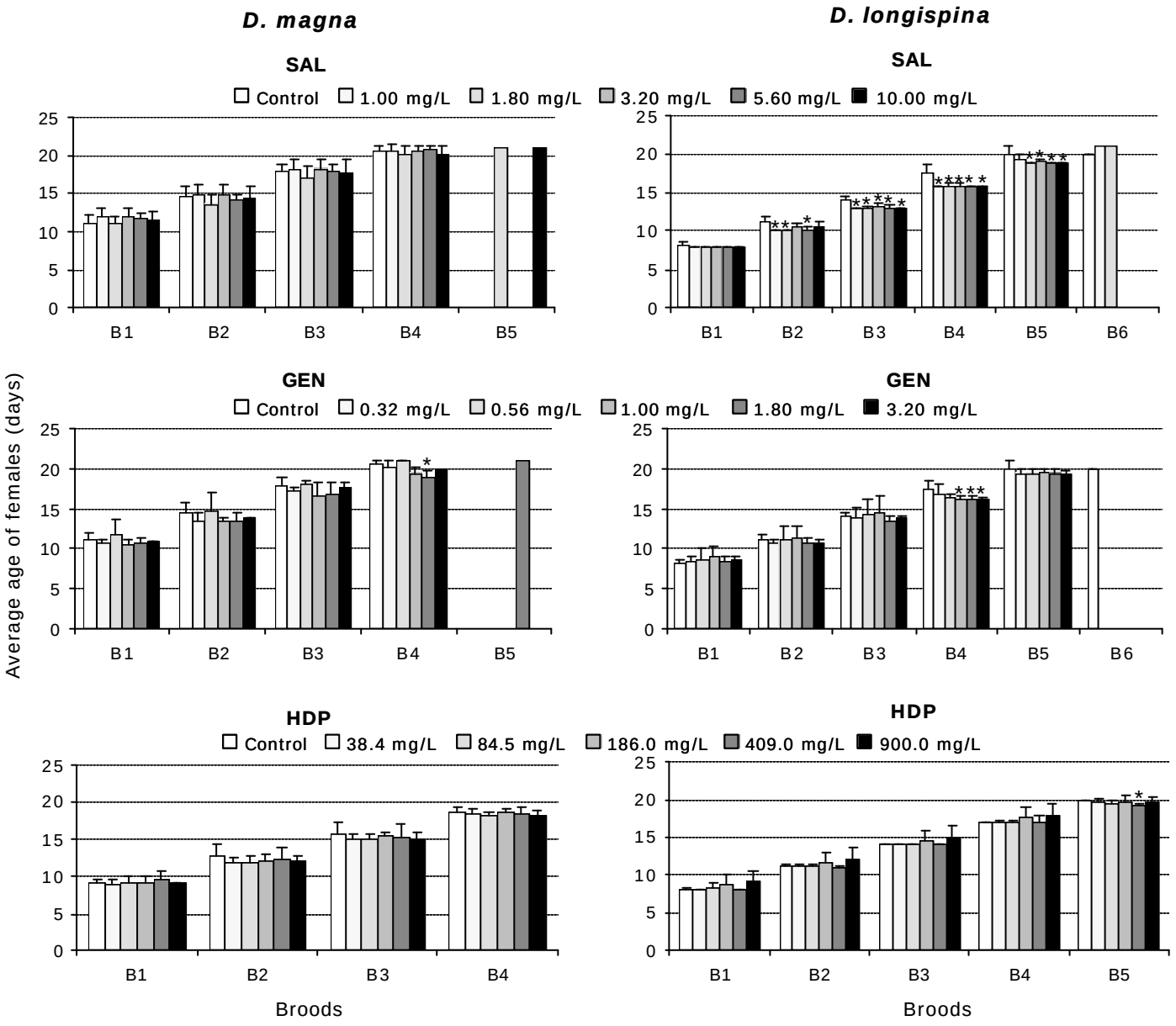

Fig. 6. Average age of females at each brood released by *D. magna* and *D. longispina*, at different concentrations of SAL, GEN and HDP. Error bars represent the standard deviation, and * indicates a significant difference from the control ($P<0.05$).

Despite this, only in B4 ($F_{[4,15]}$ = 6.05, P<0.05) for GEN treatment it was caculated a statistical difference for *D. magna*, while *D. longispina* released its offspring significantly earlier in B2 ($H_{(0.05,5)}$ = 31.42, P<0.05), B3 ($H_{(0.05,5)}$ = 40.45, P<0.05), B4 ($H_{(0.05,5)}$ = 41.73, P<0.05) and B5 ($H_{(0.05,5)}$ = 27.89, P<0.05) for SAL; in B4 ($H_{(0.05,5)}$ = 20.26, P<0.05) for GEN; and in B5 ($H_{(0.05,5)}$ = 12.78, P<0.05) for HDP.

Age at first reproduction was not significantly different from the control whatever the metabolite or species considered. Even so, the number of neonates released was affected in B1, specially for *D. longispina*, as well as their size (Fig. 7).

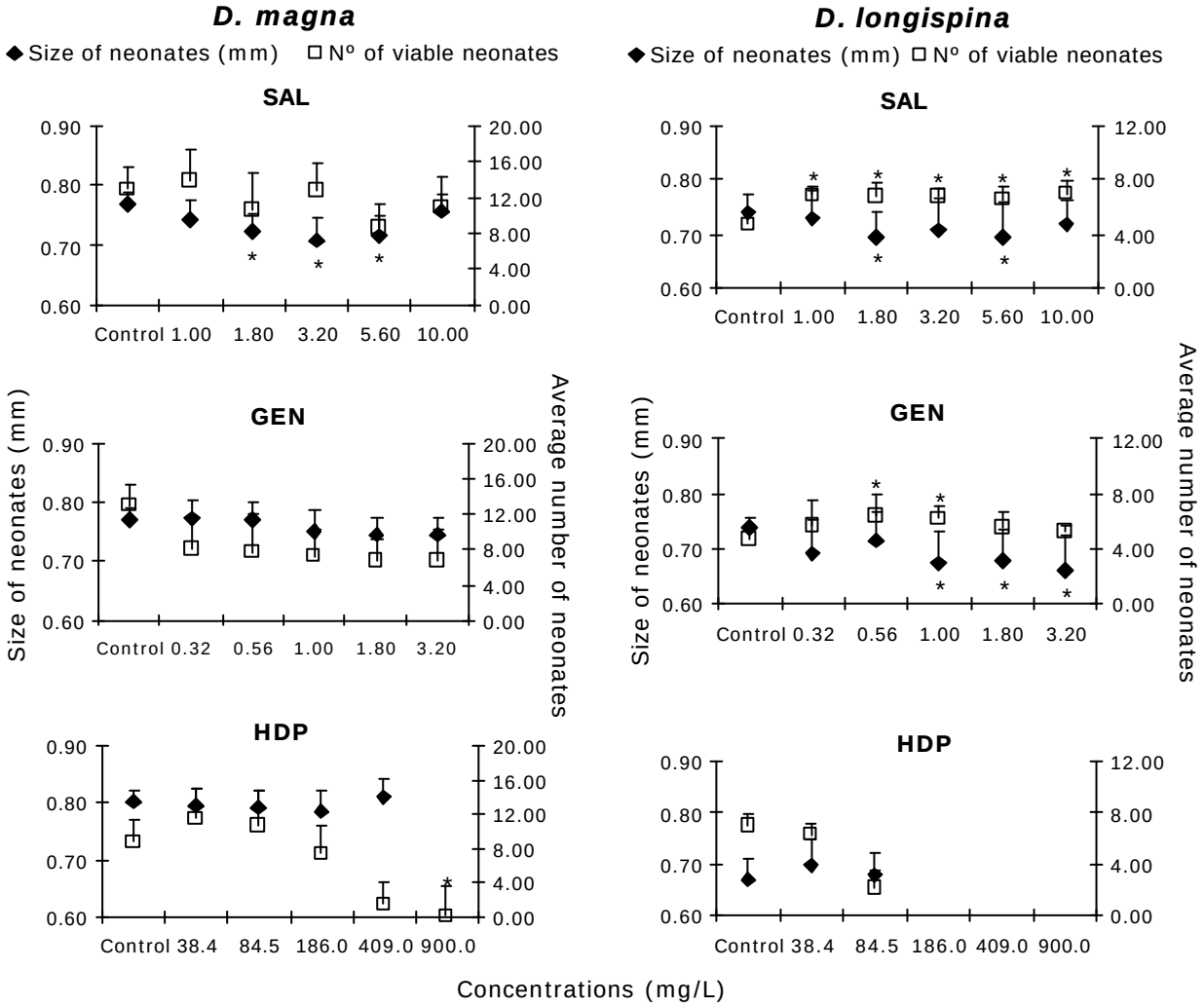

Fig. 7. Average number of neonates from the first brood *versus* their size, produced by *D. magna* and *D. longispina* at different concentrations of SAL, GEN and HDP. Error bars represent the standard deviation, and * indicates a significant difference from the control (P<0.05).

The significative reduction of *D. magna* neonates size ($F_{[5,150]}$ = 18.08, P<0.05) is in agreement with the oscillations in the number of neonates, under similar concentrations of SAL (Fig. 7). The same had occurred for the size of *D. longispina* neonates when adult females were exposed to SAL ($H_{(0.05,5)}$ =19.79, P<0.05) and GEN ($H_{(0.05,5)}$ = 34.29, P<0.05). No statistical differences were recorded for *D. magna* exposed to GEN, neither were to HDP for both species. Overall, the size of neonates tends to reduce to increasing levels of toxicant.

Growth

The population growth rate (*r*) (Fig. 8) had been affected by the three acids exposures. Despite this, while SAL significantly stimulated the *D. longispina* population growth ($H_{(0.05,5)}$ = 20.01, P<0.05) to every tested concentrations (NOEC<1.00 mg/L), GEN induced a significant decrease of *D. magna r* to the highest concentration ($F_{[5,51]}$ = 2.46, P<0.05; LOEC = 3.20 mg/L). For HDP, the population growth of *D. magna* was significantly enhanced in lower concentrations, whereas, to higher levels of acid (409.0 and 900.0 mg/L), *r* had been inhibited, due to the aforementionated increasing number of non viable offspring. This fact justifies the sudden decrease of *D. longispina r* to lower concentrations than those observed for *D. magna*.

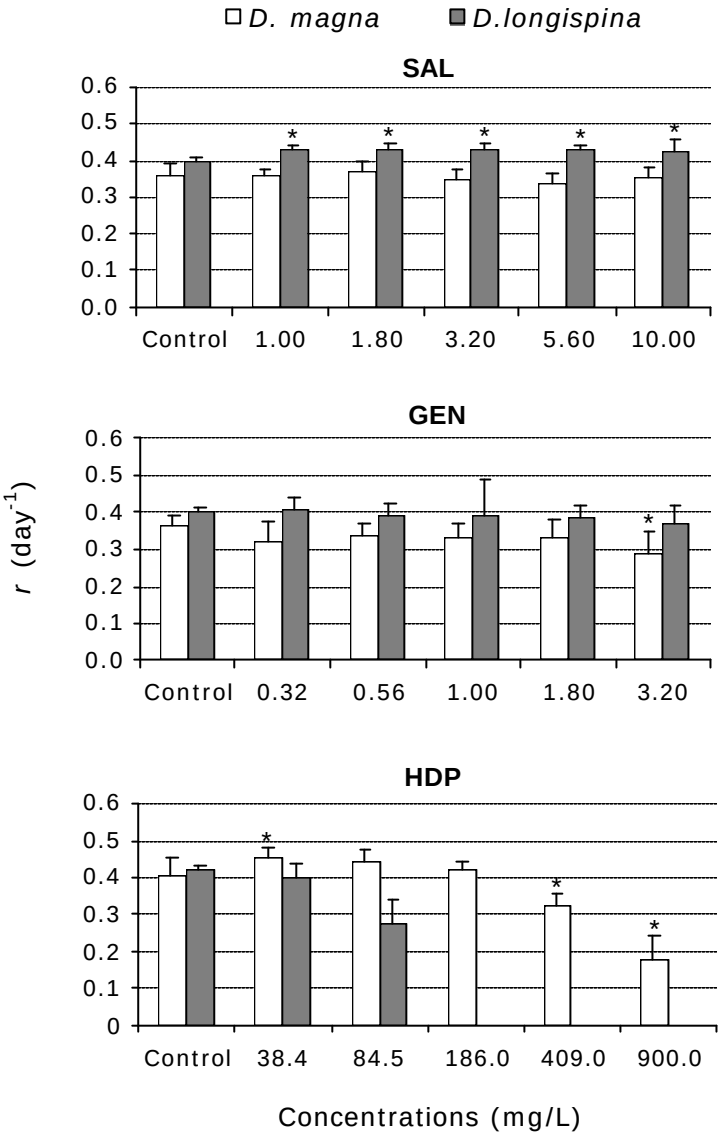

Fig. 8. Population growth rate (*r*) of *D. magna* and *D. longispina*, at different concentrations of SAL, GEN and HDP. Error bars represent the standard deviation, and * indicates a significant difference from the control ($P<0.05$).

Both SAL ($F_{[5,47]} = 2.57$, $P<0.05$) and GEN ($F_{[5,27]} = 3.45$, $P<0.05$) significantly inhibited the DGR of *D. magna*, though the LOEC of the latter (1.00 mg/L) is quite smaller than that of the former (10.00 mg/L) (Fig. 9). The growth of *D. longispina*, however, was not significantly affected by SAL ($F_{[5,50]} = 0.73$, $P>0.05$), contrary to what was noticed for GEN ($F_{[5,50]} = 3.25$, $P<0.05$; LOEC = 3.20 mg/L). No differences were recorded for HDP. Comparing the DGR responses of the two species, it is clear that *D. longispina* was less sensitive than *D. magna*, as its LOECs are higher for both SAL and GEN.

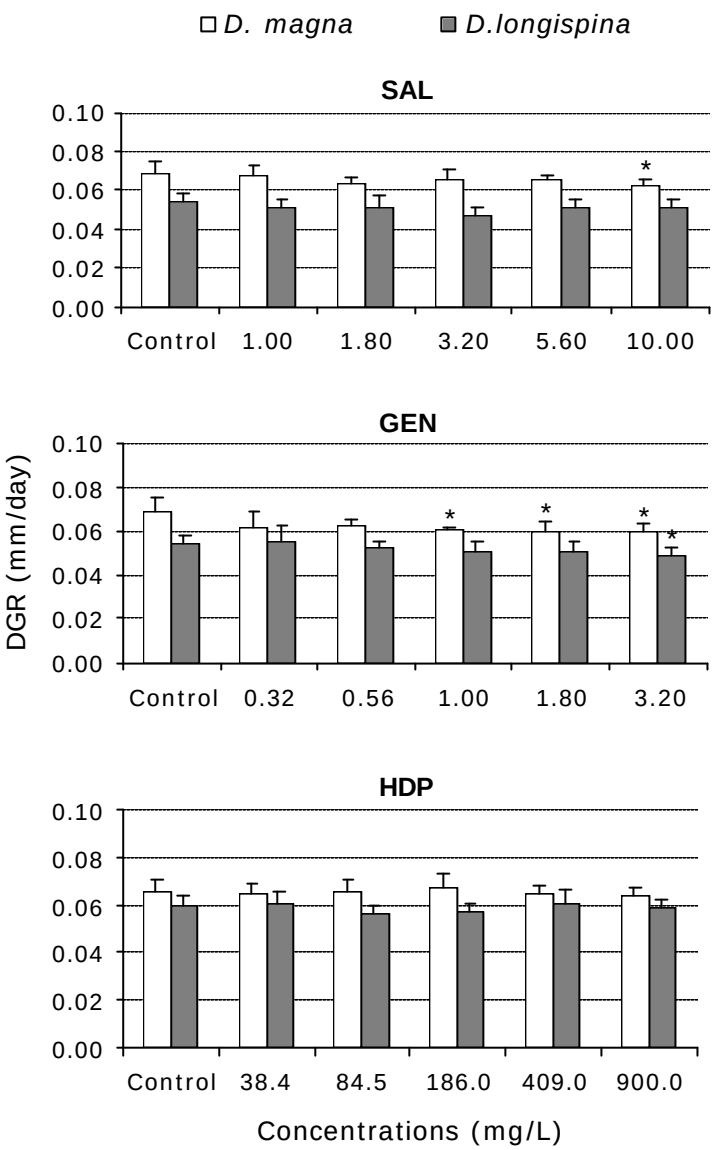

Fig. 9. Daily growth rate (DGR) of *D. magna* and *D. longispina*, at different concentrations of SAL, GEN and HDP. Error bars represent the standard deviation, and * indicates a significant difference from the control ($P<0.05$).

DISCUSSION

Public and scientific awareness about potential toxic effects of pharmacologically active compounds on non-target individuals is quite recent. Therefore, the available literature about this issue is scarce and much of the data relates to parent drugs rather than metabolites. Neverthless, the products of

biotransformation can be even more toxic than the original compound, what may constitute a serious threat to the aquatic species exposed to them.

The results attained in this study clearly show that most tested metabolites of ASA induce acute and chronic effects on non-target species.

The bioluminescence intensity of *V. fischeri* was inhibited only by SAL at the highest test solution concentration of 1800 mg/L. Backhaus *et al.* (1997) studies show that the toxicity of some specifically acting chemicals is underestimated in the 30 min acute assay. Actually, this time is too short to cover an adequate period of the cell cycle of *V. fischeri*, which may be affected by toxic substances acting on byosynthetic pathways supporting its growth and reproduction. Further studies on this specific issue would be required to know whether GEN and HDP have the ability to change biosynthetic pathways of this bacterium, hence presenting a delayed toxicity. However, this could be a possible explanation for the lack of toxicity of these metabolites for *V. fischeri*. Meanwhile, the obtained EC_{50} was at least one order greater than that published by Henschel *et al.* (1997) (90 mg/L) for SAL; and higher than 445.48 mg/L for the same test solution concentration of ASA (unpublished data). As it was already reported by Kaiser (1998), the similarity between the EC_{50}s of both bioluminescence inhibition and *D. magna* acute immobilization assays assume a significant correlation between them, allowing exploitations from one to another. However, in this study, *V. fischeri* EC_{50} for SAL was below those calculated for the two *Daphnia* species.

The sequence of decreasing acute toxicity of ASA metabolites for both cladocerans species was: GEN > SAL > HDP, being the latter the least toxic as it did not induce immobilization of daphnids to concentrations below its solubility limit. The autochthonous daphnid (*D. longispina*) was the most sensitive species, presenting lower acute EC_{50} values for SAL (1147.57 mg/L) and GEN (342.41 mg/L) than *D. magna* (1945.32 and 402.55 mg/L, respectively). Some studies indicate that *D. magna* is more tolerant as its larger size gives it a smaller surface area-to-volume ratio as compared to smaller species (*e.g.*, *D. pulex*), which might lead to proportionally lower accumulation of toxic compounds (Lilius *et al.*, 1995) than, for instance, for *D. longispina*.

Other studies focusing on acute toxic effects of pharmacologically active substances showed that SAL had immobilized *D. magna* at 118 mg/L (Henschel *et al.*, 1997), which is far below the EC_{50}s herein determined for both *D. magna*

and *D. longispina*. Neverthless, it was already reported the same response induced by ASA at 1469 mg/L (Lilius *et al.*, 1995) and 1293.05 mg/L (unpublished data) for *D. magna*; while for *D. longispina* the ASA EC_{50} was 647.31 mg/L (unpublished data). These results suggest that SAL has an approximate acute toxic effect of that of ASA, although the former, apparently, is still less toxic than the latter. Further experiments carried out with SAL acid revealed its toxicity on the mortality (EC_{50} = 37 mg/L) and decrease pulse rate (EC_{50} = 50 mg/L) of fish embryos (Henschel *et al.*, 1997). Data related with toxic effects of GEN and HDP were not found in the available literature. However, considering the EC_{50}s of ASA already mentioned, it seems obvious that its GEN metabolite is much more toxic than the parent compound is for the two cladocerans species.

In the conducted chronic assay, GEN had fairly constrained the survival of *D. magna* individuals, whereas HDP was responsible for the highest mortalities recorded on *D. longispina*. Actually, although a dose-response pattern is not apparent, the great part of mortalities observed had occurred to ?10 days of exposure, and almost all of the individual replicates had released at least one clutch before dying. This behaviour seems to reflect the effort developed by the organisms to survive until assure that population would not be extincted at all.

Long-term exposures to the three metabolites of ASA had significantly impaired the normal reproduction and growth of *D. magna* and *D. longispina*. A dose-response relationship was defined for the total number of viable offspring produced per female for both species exposed to all toxicants. GEN was the strongest inhibitor of the reproductive output either of standard or autochthonous daphnids (LOEC = 0.56 mg/L). SAL induced an intermediate inhibitory effect, which was significant at the LOEC of 10.00 mg/L for *D. longispina*, while *D. magna* was not significantly affected (NOEC > 10.00 mg/L). Both cladocerans had significantly reduced the number of viable offspring when subjected to HDP, although the autochthonous species was again the most sensitive one, suffering a drastic reduction on this parameter at 84.5 mg/L, which was totally inhibited from 186.0 mg/L of HDP.

Neverthless, this last trend was substituted by the increasing number of malformed neonates and aborted eggs released under higher concentrations of HDP during the test. Unviable offspring was mainly represented by aborted eggs, though *D. magna* had produced some abnormal neonates at the two highest

concentrations. In relation to the number of aborted eggs, a positive correlation with HDP dose could be observed for *D. magna*. Although this pattern had been followed by *D. longispina* under lower concentrations of HDP, for the highest tested levels of 409.0 and 900.0 mg/L, the number of produced eggs had diminished, reflecting a slight reproductive inhibition. Several authors (*e.g.*, Baird *et al.,* 1991; Klüttgen *et al.*, 1996; Guilhermino *et al.*, 1999; Trubetskova and Lampert; 2002) verified that sodium bromide and 3,4-dichloroaniline (DCA) caused an increased level of abnormal embryonic development and abortion, and hence reduced the total number of viable offspring released. Baird *et al.* (1991) demonstrated that egg production was unaffected by chronic exposures to both chemicals, which is conform with *D. magna* behaviour, though unlike that of *D. longispina* to higher levels of HDP. They hypothesized that the effect of DCA on reproduction was due to direct poisoning of developing embryos in the brood chamber and not on eggs in the ovary. Future experiments should examine the development of parthenogenetic eggs in order to precise HDP action. Actually, this molecule is quite big (Fig.1), which probably makes it difficult to penetrate the carapace of the mother and, thus, may affect eggs in the ovary after bioaccumulation.

Considering the average number of viable offspring produced in successive broods for both species it is evident a decrease pattern to increasing concentrations of each metabolite, mainly significant for *D. longispina* and GEN exposures. However, *D. longispina* fecundity was significantly stimulated in B1 to all tested concentrations of SAL and to lower levels of GEN. Neverthless, for *D. magna*, a low-dose stimulation and a high-dose inhibition had occurred to HDP treatment, though not significantly different from the control. An hormetic response could be related to the observed trend for GEN, as it was already observed for *D. longispina* exposed to ASA (unpublished data). In spite of this, the other stimulatory effects detected cannot be explained as an hormetic effect, once they do not cover its typical characteristics referred by Calabrese (2002).

Thus, it is quite clear that first reproduction, an ecologically relevant life-stage to cladocerans maintenance in ecosystems, is affected by these bioactive substances. Comparing the number of viable neonates and is their size, a subtle negative correlation is apparent, specially for *D. longispina*, *i.e.*, the greater the number of produced neonates is the smaller their size. Bradley *et al.* (1991) had actually demonstrated a similar correlation relatively to *D. magna* egg number

and size, although unfavourable conditions corresponded to daphnids starvation. Notwithstanding, it was also common to observe a decreasing number of viable offspring with smaller size released by both species exposed, in particular, to higher concentrations of SAL and GEN. Considering that *Daphnia* sp. is a biological system, in which energy supply mainly provided by food ingestion is canalized to its maintenance, activity and reproduction, any toxic stress reducing the ingestion or assimilation rates, or even acting directly upon its maintenance, may mislead the energy allocation, hence altering the normal reproductive investment to the production of eggs (Soares, 1989; Baird *et al.*, 1990). Thereby, the evaluation of mass transfer to offspring along the successive reproductions would be required to better withstand that hypothesis.

SAL and GEN were potential inhibitors of both cladocerans DGR, being the latter the strongest inhibitor at lower doses. Neverthless, daphnids did not reflect the same sensitiveness and the autochthonous one was the most tolerant species for both toxicants, as its NOECs and LOECs assumed lower values. In spite of this, HDP had not changed both daphnids DGR.

Until now, the effects of ASA metabolites have been assessed at individual-level responses. Overall, Forbes and Calow (1999) came to the conclusion that *r* is a better measure of responses to toxicants than the individual-level effects, because it integrates potentially complex interactions among life-history traits (*e.g.*, survival, reproductive output, age at each brood release), providing a more relevant measure of ecological impact. The autochthonous species *r* assumed higher values than those determined for the standard species, for both SAL and GEN treatments, which is due to its normal shorter life-cycle represented by an earlier age at maturation and a smaller period between broods. In spite of this, in the presence of SAL, the *r* of *D. longispina* had significantly raised to increasing concentrations, contrary to what happened for *D. magna*. This result reflects not only the low mortalities recorded for the autochthonous cladoceran (30% at 1.00 mg/L and 10% at 3.20 mg/L), but also the significantly earlier release of clutches by females exposed to SAL. On the other hand, GEN induced the *r* decrease for both standard and autochthonous daphnids to increasing levels, though only significantly different from the control for *D. magna*, at the highest level of compound (LOEC = 3.20 mg/L). This conforms with the high mortalities and the fecundity inhibition (LOEC = 0.56 mg/L) induced by GEN on the standard species, thereafter reducing its

population growth. For HDP, a low-dose stimulation followed by a high-dose inhibition was defined for *D. magna*, while *D. longispina* showed a decreasing trend of its *r*, which became null at 186 mg/L. In this case, the reduced survivorship, the abortion of eggs and the decreasing reproductive output had severely constrained the fitness of *D. longispina*, which was more sensitive to HDP than the standard cladoceran.

Overall, the autochthonous daphnid was generally more sensitive than the standard species, although some noteworthy exceptions could be observed in the chronic assay: in the presence of SAL, *D. longispina* revealed a developed ability to adjust its life-history strategy in order to maximize its population growth and subsequent persistence, which may reflect an increased phenotypic plasticity of autochthonous species. Moreover, *D. magna* somatic growth was more affected by chronic exposures to SAL and GEN than that of *D. longispina*, which might had diminished the growth rate during females juvenile stage, therefore inhibiting *D. magna* normal reproduction and decreasing its fitness (Hanazato, 1998; 2001), hence constraining its survival. This is reflected by the evoked decrease of its population growth, which was statistically different from the control under GEN exposures. In summary, ASA metabolites affected different life-history parameters of both cladocerans and thus influenced life-history strategy in different ways. Therefore, a generalization of toxicant effects on different species, even if closely related, remains difficult, as it was already concluded by Klüttgen *et al*. (1996).

Thus, reproduction and growth rate of cladocerans were susceptible to SAL and GEN effects; whereas for HDP, reproduction was found to be the most sensitive parameter, therefore strongly affecting the intrinsic population growth of both species.

In spite of the elevated EC_{50}s, NOECs, and LOECs obtained in the acute and chronic assays, the concentrations of these compounds in natural waters are much lower or even nule (for HDP). However, considering that they are bioactive substances designed to induce specific effects, one cannot exclude the possibility that first warning responses at a lower organizational level are likely to occur on non-target individuals (Ternes, 1999). Therefore, the assessment of physiological and biochemical changes in normal mechanistic processes, upon low dose long term exposures would be an interesting issue to study in future research works on cladocerans, an important key group in shallow lake ecosystems.

Furthermore, the aquatic organisms are typically not exposed to single substances but rather simultaneously to multiple mixtures of xenobiotics (Backhaus *et al.*, 2000; Faust *et al.,* 2000). Therefore, a desirable ecologically relevant study would include the analysis of the toxicity level of a complex chemical mixture on non-target species, inferring possible acute and/or chronic effects due to synergistic or antagonist interactions among pharmacological residues.

CONCLUSION

In general, the sequence of decreasing toxicity of ASA metabolites was GEN > SAL > HDP. The bioluminescence intensity of *V. fischeri* was inhibited only by SAL at the highest test solution level. Moreover, HDP did not present acute toxicity to the test organisms utilized below its solubility limit. In opposition, chronic exposures to HDP had drastically impaired both autochthonous and standard cladocerans reproduction, causing egg abortion and production of abnormal neonates, hence reducing *Daphnia* fitness. On the other hand, SAL and GEN affected either animals normal reproduction or their growth.

D. longispina was, generally, the most sensitive cladoceran, although it was able to increase its population growth under long term exposures to SAL acid in relation to *D. magna*. Furthermore, *D. magna* DGR was more affected than that of *D. longispina*, which might have also constrained its reproductive output.

Overall, the determined effect concentrations were above the levels detected in environmental samples. Neverthless, long term exposures to low levels of pharmacological active substances, usually present as complex mixtures, may induce slight physiological and biochemical changes in non-target organisms, that can cumulate over time, and thus manifestate delayed effects on individual- and even population-level traits.

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Capítulo IV

Discussão geral

DISCUSSÃO GERAL

Este estudo torna-se inovador na medida em que integra e compara a análise de possíveis efeitos em organismos padrão e autóctones, decorrentes de exposições agudas e crónicas a compostos farmacológicos. Para além disso, não só a toxicidade do princípio activo de um medicamento foi avaliada, mas também a dos seus principais produtos de metabolização, a qual é, muitas vezes, desprezada.

Neste sentido, os ensaios ecotoxicológicos desenvolvidos, para as quatro substâncias farmacológicas consideradas, provaram que tanto o composto parental, como grande parte dos seus metabolitos, induzem efeitos agudos e crónicos nos organismos de teste utilizados.

Os ensaios agudos realizados em ambos os cladóceros evidenciaram a sua imobilização de acordo com o grau de toxicidade decrescente GEN > ASA > SAL > HDP, em que este último não apresentou toxicidade para concentrações inferiores ao seu limite de solubilidade. No entanto, os CE50s (concentração que provocou um efeito em 50% da população de teste) calculados para a espécie autóctone (*D. longispina*) foram inferiores aos obtidos para espécie padrão (*D. magna*), reflectindo a maior sensibilidade da primeira a exposições agudas daqueles compostos.

Kaiser (1998) referiu a existência de uma correlação significativa entre respostas agudas de *D. magna* e da bactéria marinha bioluminescente *V. fischeri*, inferindo acerca da possível extrapolação de resultados de uma a partir da outra. Contudo, somente ASA e SAL inibiram a bioluminescência de *V. fischeri*, apesar dos valores de CE50 e o grau de toxicidade de cada composto serem relativamente concordantes com os obtidos no ensaio de imobilização dos cladóceros, mas mais próximos dos de *D. longispina*.

Para *V. fischeri*, a aparente ausência de toxicidade de GEN e HDP pode dever-se ao curto período de teste (30 min.). Backhaus *et al.* (1997) constataram que alguns compostos químicos actuam especificamente ao nível de vias biossintéticas reguladoras do crescimento e reprodução desta espécie. Uma vez que 30 min. é inferior ao tempo necessário para completar um ciclo de vida desta bactéria é possível que as toxicidades de GEN e HDP estejam a ser subestimadas. Para apoiar esta hipótese, outros estudos ter-se-iam de

desenvolver para elucidar a acção destes dois metabolitos no ciclo de vida de *V. fischeri*.

Relativamente aos testes crónicos desenvolvidos em ambos os dafnídeos, verificou-se que quase todos os compostos afectavam a sua reprodução e crescimento normais. De uma forma geral, GEN foi o inibidor mais forte dos parâmetros de ciclo de vida analisados, seguido de ASA, SAL e HDP.

No que diz respeito à reprodução, *D. longispina* tendeu a ser a espécie mais sensível, embora, na presença de ASA e GEN, os dois cladóceros tenham apresentado CEOs (concentração para a qual se verificou um efeito nos organismos de teste) semelhantes. De acordo com Lilius *et al.* (1995), alguns estudos mostraram que *D. magna* é normalmente mais tolerante, já que o seu maior tamanho implica uma razão área de superfície/volume inferior, comparativamente com espécies menores (*e.g.*, *D. pulex*). Assim, a acumulação de compostos tóxicos por *D. magna* poderá ser proporcionalmente inferior à de, por exemplo, *D. longispina*, tornando-a menos sensível à acção de xenobióticos.

Além disso, Hanazato e Hirokawa (2001) mostraram que a sensibilidade dos dafnídeos a um composto químico é negativamente correlacionável com o tamanho do corpo (TC), numa regressão linear. Explicaram, então, que se devia ao equilíbrio estabelecido entre a taxa de incorporação do xenobiótico (TC^2) e respectiva taxa de destoxificação (TC^3), sendo esta condicionada pela quantidade de enzimas destoxificantes presentes nos animais. Assim, indivíduos de maior tamanho e massa serão, à partida, mais tolerantes do que os que possuem dimensões mais reduzidas. Não obstante, uma análise ao nível da bioquímica e fisiologia celulares teria de ser levada a cabo, por forma a elucidar o comportamento destes tóxicos nos organismos em questão.

Embora HDP não tenha revelado toxicidade aguda para os organismos de teste, o mesmo não sucedeu para exposições crónicas, cuja acção afectou a reprodução normal dos cladóceros, induzindo descendência não viável. Ou seja, o HDP promoveu a produção de neonatos indiferenciados e, principalmente, o aborto de grande parte dos ovos produzidos. Este efeito foi particularmente notório na espécie autóctone, cuja sobrevivência, num estado mais avançado, foi afectada. Por outro lado, a espécie padrão mostrou-se mais tolerante, sendo a responsável pela libertação da maior parte dos juvenis inviáveis registados durante o ensaio. Vários autores (*e.g.* Baird *et al.,* 1991; Klüttgen *et al.*, 1996;

Guilhermino *et al.*, 1999; Trubetskova e Lampert, 2002) referiram a produção de descendência inviável em *Daphnia* sp. induzida por 3,4-dicloroanilina. Baird *et al.* (1991) apontaram a possibilidade da acção do tóxico em *D. magna* incidir directamente nos embriões em desenvolvimento no marsúpio e não ao nível dos ovos no ovário, até porque a produção destes não sofreu qualquer alteração. Outras experiências deveriam ser conduzidas para perceber a acção de HDP no desenvolvimento de ovos partenogénicos, pois, a composição e estrutura químicas de cada composto condicionam o seu comportamento toxicocinético e efeitos negativos específicos nos organismos a eles expostos.

Baird *et al.* (1991) afirmaram ainda que nem todos os compostos actuam da mesma forma: alguns podem penetrar a carapaça da mãe e afectar os ovos no ovário, enquanto outros podem, indirectamente, influenciar a fecundidade ao inibir o crescimento da fêmea. Na verdade, a presença de ASA, SAL e GEN reduziu a fecundidade das fêmeas em todas as ninhadas, excepto na primeira (em que, ASA e GEN estimularam a fecundidade a baixas concentrações e inibiram-na a altas concentrações, enquanto SAL estimulou a produção de neonatos em todas as concentrações testadas). Em adição, o tamanho dos juvenis da primeira ninhada foi, de uma maneira geral, significativamente menor para concentrações de tóxico superiores. Estes dois factores sugerem um possível impacto a outro nível que não directamente nos embriões em desenvolvimento no marsúpio, mas provavelmente, de uma forma indirecta, durante o período de formação dos ovos.

Corroborando aquela hipótese, Hanazato (1998; 2001) indicou que o número de neonatos produzidos por ninhada não era directamente afectado pelo tóxico testado. Antes, estava condicionado pelo TC da fêmea, dependendo este do seu crescimento durante o estado juvenil. Deste modo, tendo em conta que a taxa de crescimento diário foi afectada tanto para *D. magna* como para *D. longispina*, sujeitas a ASA, SAL e GEN, é provável que o seu crescimento normal, enquanto juvenis, tenha sido alterado. Ou seja, a redução do crescimento das fêmeas pode promover a libertação de um menor número de neonatos.

Na verdade, *Daphnia* é um sistema energético, cuja sobrevivência, actividade e reprodução são funções asseguradas pela energia normalmente fornecida pelo alimento. Por isso, qualquer *stress* químico, passível de reduzir as taxas de ingestão ou assimilação, ou capaz de afectar a sobrevivência do organismo, pode alterar o seu processo normal de investimento de energia,

nomeadamente, na reprodução, influenciando o desempenho e fecundidade das fêmeas (Baird *et al.*, 1990).

Embora a taxa de crescimento intrínseco da população (TCI) englobe e integre variáveis parciais do ciclo de vida dos cladóceros (*e.g.*, sobrevivência e fecundidade cumulativa) (Forbes e Calow, 1999), nem sempre reflectiu a mesma tendência das respostas observadas em cada parâmetro individual. A TCI da espécie autóctone, ao contrário de *D. magna*, foi estimulada na presença de ASA e SAL, evidenciando uma plasticidade fenotípica mais desenvolvida perante certas situações desfavoráveis, no sentido de garantir a permanência da sua população no ecossistema. Verifica-se, portanto, que é aconselhável a introdução de espécies autóctones como organismos de teste na avaliação da toxicidade de compostos químicos, por forma a obter uma análise com maior relevância ecológica. No entanto, compostos com diferentes estruturas e propriedades químicas podem induzir diferentes sensibilidades nas espécies em estudo. Assim, tanto GEN como HDP, ao contrário das outras substâncias, reduziram a TCI de ambos os dafnídeos o que, neste caso, está de acordo com a observada inibição das respostas parciais.

Apesar das concentrações testadas estarem muito acima das vulgarmente detectadas no ambiente, não se deve excluir a hipótese de, a longo prazo, ocorrerem alterações muito subtis a um nível de organização biológico inferior (*e.g.*, bioquímico), cuja acumulação pode manifestar efeitos fisiológicos e morfológicos profundos (Daughton e Ternes, 1999). Aliás, *Daphnia* spp. possuem receptores moleculares, semelhantes aos que existem em espécies de vertebrados, sobre os quais substâncias farmacologicamente activas têm a capacidade de actuar (Barry e Stoopman, 2000). Neste sentido, compostos deste tipo são potenciais indutores de alterações dos mecanismos celulares de organismos não alvo, principalmente pelo facto de se encontrarem em misturas complexas no ambiente aquático. Por esta razão, teria todo o interesse realizar o estudo ecotoxicológico da mistura de substâncias bioactivas no ciclo de vida e dinâmica populacional de cladóceros, organismos chave em ecossistemas de água doce temperados.

Em suma, as substâncias medicamentosas são passíveis de induzir efeitos agudos e crónicos em indivíduos não alvo. No entanto, neste estudo não só o composto parental (ASA) apresentou toxicidade para os organismos aquáticos

utilizados, mas também os seus produtos de metabolização, principalmente o metabolito GEN revelou ser o composto mais tóxico. Pelo contrário, HDP foi o menos tóxico. Apesar deste não ter induzido efeitos agudos nos organismos de teste, inibiu drasticamente os parâmetros reprodutivos e populacionais de cladóceros sujeitos à sua exposição crónica. Desta forma, verifica-se que a aplicação de ensaios agudos em análise de risco ambiental de compostos a serem regulamentados pode ser insuficiente, já que exposições a longo prazo promovem alterações no ciclo de vida dos indivíduos, comprometendo, assim, a sua permanência e subsequente equilíbrio dos ecossistemas aquáticos.

Para além disso, a utilização de espécies autóctones, neste tipo de estudos, permite avaliar de uma forma mais realista, o comportamento individual e populacional de organismos sujeitos a condições desfavoráveis no seu ambiente natural.

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